THE BIOCHEMICAL JOURNAL

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CORRIGENDA

Action of streptomycin on *Vibrio cholerae*

By C. R. Krishna Murti

Volume 76 (1960)

p. 366, legend to Fig. 1, line 2

for 33 g. of dihydrostreptomycin *read* 33 μg. of dihydrostreptomycin/ml.

The metabolism of isolated rat-liver nuclei

By K. R. Rees and G. F. Rowland

Volume 78 (1961)

p. 90, Table 1, line relating to nuclei (no.)

for 10^5 *read* 10^6

p. 90, Table 2 to read as follows:

	$\mu\mu$g./nucleus	μg./mg. of nuclear nitrogen
Nitrogen	16·4 (15·3–17·1)	—
Phosphorus		
DNA	0·69 (0·60–0·76)	45 (39–52)
RNA	0·67 (0·58–0·72)	44 (42–48)
Phospholipid	0·59 (0·55–0·74)	37 (34–44)

The autodegradation of ribonucleoprotein in *Escherichia coli*

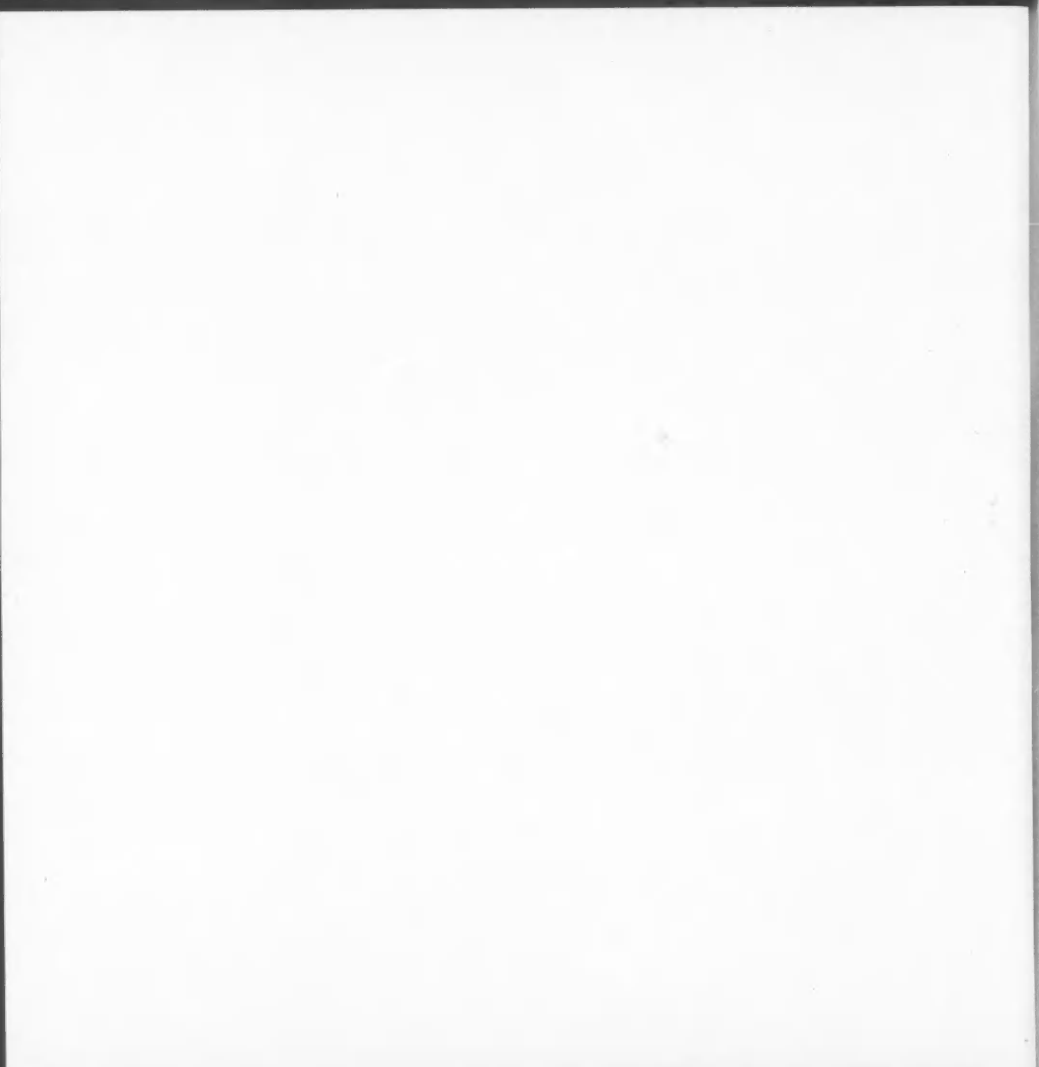
By H. E. Wade

Volume 78 (1961)

p. 457, column 1, 4 lines from end

for 2·8 l. of soln. *A*, 750 ml. of soln. *B* and 1 l. of soln. *C*

read 2·8 l. of soln. *A*, 400 ml. of soln. *B* and 800 ml. of soln. *C*



Biochem. J. (1961) **79**, 1

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The Association of ^{14}C-Labelled Vitamin D_2 with Rat Serum Proteins

BY KAREEN J. INNES CHALK AND E. KODICEK
Dunn Nutritional Laboratory, University of Cambridge and Medical Research Council

(*Received* 13 *October* 1960)

Separation of serum by electrophoresis has shown that certain lipids, found in blood, have the same electrophoretic mobilities as some of the proteins. It is believed that this is due to the attachment of the lipids to the serum proteins in transport by the blood stream. Vitamin A alcohol was found to be carried by α_1-globulin (Garbers, Gillman & Peisach, 1958, 1960); carotene was located mainly with β-globulin, but also with albumin and the α-globulins in human serum (Crook & El-Marsafy, 1954) and with albumin in bovine serum (Erwin, Varnell & Page, 1959); corticosteroids appeared to be associated with albumin (Daughady, 1956, 1958). The transport of vitamin D by human serum has been studied (Morgan, Thomas, Haddock & Howard, 1958; Thomas *et al.* 1959). These authors separated, by electrophoresis on starch, the serum proteins from patients given high doses of vitamin D, and tested the protein fractions for antirachitic activity. Owing to the low level of the vitamin in blood they had not enough material to subdivide the protein fractions, but they were able to show that it can all be found in the albumin and α-globulin regions. Similar results were obtained from human serum incubated *in vitro* with vitamin D.

By using ^{14}C-labelled vitamin D_2 it has been possible to analyse smaller regions of the starch block after electrophoresis and to study thereby the distribution of the vitamin with respect to protein in more detail. The serum used in these experiments was obtained from rats and incubated *in vitro* with ^{14}C-labelled vitamin D_2. For preliminary communication, see Chalk & Kodicek (1960).

MATERIALS

Serum. About 45 ml. of unhaemolysed blood was obtained by heart puncture from 6-month-old, male, stock rats. After an hour at room temperature the blood was centrifuged and the serum used on the same day.

Vitamin D_2. Uniformly ^{14}C-labelled vitamin D_2 (specific activity, 0·4 μc/mg.), prepared by Kodicek (1955), was dissolved in propylene glycol (0·5 mg./ml.).

Buffer. Barbiturate buffer, pH 8·6, was used for electrophoresis: 10·3 g. of sodium diethylbarbiturate and 1·84 g. of diethylbarbituric acid (British Drug Houses Ltd.) per litre.

Starch. Potato starch (British Drug Houses Ltd.) was Soxhlet-extracted for 6 hr. with diethyl ether to decrease its fat content, which would otherwise have contaminated the lipids extracted after electrophoresis.

Organic solvents. A.R.-grade diethyl ether, propan-1-ol and light petroleum (b.p. 40–60°) were purified by distillation. The other organic solvents used were A.R.-grade methanol, acetone (British Drug Houses Ltd.) and absolute ethanol (The Distillers Co. Ltd.).

Folin–Ciocalteu reagent. The Folin–Ciocalteu reagent for tyrosine (British Drug Houses Ltd.) was kept in a dark bottle in the refrigerator and used within 2 months of preparation.

METHODS

Incubation of serum with vitamin D_2

For each experiment 20 μl. of ^{14}C-labelled vitamin D_2 (10 μg.) in propylene glycol was added slowly, with shaking, to 4 ml. of serum. The serum was incubated at 37° for 30 min. and then separated by electrophoresis on starch.

Electrophoresis on starch

The method used was a modification of that devised by Kunkel & Slater (1952*a*). Starch (150 g.) was mixed to a stiff paste with barbiturate buffer and poured into a rectangular metal mould (21 cm. × 8 cm. × 1·5 cm.) lined with filter paper. After drying for 2–3 hr. at room temperature, the block was turned out on a glass plate and the paper removed. The serum, after incubation with vitamin D_2, was added to the block by mixing it with dry starch and pouring it into a 1 cm. wide trough in the block, 4·5 cm. from the cathode end. At each side of the trough 0·5 cm. margins were left free from serum to reduce streaking at the edges. A small bromophenol-blue marker was put in the middle of the trough containing the serum sample, to follow the progress of electrophoresis. Finally the block was covered with a second glass plate. It was equilibrated in a Shandon electrophoresis tank at 7° for 3 hr. in a refrigerator and then run at 400 v and 32 mA for 11 hr. During the run the temperature increased only by 5°.

Analyses of the block

Two different procedures were used in the segmenting of the starch block for analysis. The blocks were cut, at right angles to their lengths, into 1 cm. segments. In Expt. 1, each segment was cut into three pieces of 3, 1 and 3 cm. widths, and dried over P_2O_5. The 0·5 cm. margins on each side were discarded. The two 3 cm. pieces were used for vitamin D analysis; two different extraction procedures were used as described below. The 1 cm. pieces were used for protein analysis; they were extracted with 3 ml. of saline (0·9% NaCl soln.) and centrifuged, and 2 ml. was taken for the estimation of protein.

In Expt. 2, the set of 1 cm. segments was not further subdivided, but each whole segment was extracted successively by the three procedures described below. Each segment was dried over P_2O_5 and shaken with 12 ml. of 0·9 % NaCl soln. and centrifuged. A sample (1 ml.) of the supernatant was taken for protein analysis and 5 ml. for measurement of saline-extractable ^{14}C-labelled material. The remainder, containing the starch, was extracted with light petroleum. Finally it was re-extracted with ethanol–diethyl ether (3:1, v/v), after being boiled with methanol as described below. Each of the fractions was analysed for vitamin D by measurements of radioactivity, paper chromatography and biological assay.

Protein analysis. The samples of the NaCl extracts reserved for protein analysis were assayed for protein by the method of Peters & van Slyke (1932). Each sample (equivalent to one-twelfth of each segment) was added to 25 ml. of water, followed by 2 ml. of 5 N-NaOH and 3 ml. of Folin–Ciocalteu reagent, and the solution was made up to 50 ml. After 5–10 min. the absorption of the solution was measured photometrically with a Wratten filter no. 70. A calibration curve was made with bovine serum albumin (Armour Laboratories Ltd.) in concentrations of up to 5 mg./ml. A serum albumin standard was included in each set of determinations and was found to agree with the calibration curve.

Extraction of vitamin D

(*a*) Saline extraction. In Expt. 1, one of the 3 cm.-wide pieces of each segment was shaken with 6 ml. of 0·9 % NaCl soln. and centrifuged, and a 3 ml. sample of the supernatant taken. This was heated with 3 ml. of methanol for 3 min. with a cold finger condenser and extracted three times with 5 ml. portions of light petroleum. The extracts were combined and evaporated to dryness under reduced pressure. In Expt. 2, 5 ml. samples of the supernatant mixed with 5 ml. of ethanol were extracted in the same way.

(*b*) Extraction with light petroleum. In Expt. 1, the other 3 cm.-wide piece of each segment was suspended in 3 ml. of ethanol and extracted three times with 5 ml. of light petroleum. In Expt. 2, the remaining 6 ml. of NaCl extract containing starch was extracted three times with 5 ml. of light petroleum. The combined extracts in each experiment were evaporated to dryness under reduced pressure.

(*c*) Boiling with methanol, followed by ethanol–diethyl ether extraction. This method of extraction was used only on the segments from Expt. 2. Each segment was refluxed with 80 ml. of methanol for 2 hr. to facilitate the subsequent extraction with ethanol–diethyl ether. The methanol was removed by evaporation under reduced pressure, and the starchy material dried over P_2O_5 and Soxhlet-extracted for 5 hr. with ethanol–diethyl ether (3:1, v/v). The extract was evaporated to dryness under reduced pressure.

Estimation of vitamin D

(*a*) Radioactivity measurements. All the various fractions from all the segments in both experiments were counted with a thin end-window Geiger–Müller tube. They were dissolved in 0·1 ml. of ethanol and 0·05 ml. of each was put on a planchet. The results were corrected for self-absorption and expressed in μmc. After being counted, the plated samples were transferred with diethyl ether to the residual solutions and evaporated to dryness.

(*b*) Reversed-phase paper chromatography, by the method of Kodicek & Ashby (1954). In Expt. 1, the fractions obtained from each experiment were redissolved in 0·1 ml. of ethanol; 0·05 ml. of each was streaked on a 2·5 cm.-wide strip of paraffin-impregnated, Whatman no. 2 paper. A 10 μg. spot of unlabelled vitamin D_2 was applied to each strip as a marker. The strips were developed by ascending chromatography in methanol–propan-1-ol–water (16:3:1, by vol.). The position of the vitamin D_2 was located by the absorption of the marker when viewed with a Hanovia short-wave (256 mμ) ultraviolet lamp. Radioautographs were made by leaving the strips in contact with Kodirex X-ray film for 3 weeks. Those strips which gave positive radioautographs were counted with a thin end-window Geiger–Müller tube.

In Expt. 2, the saline, as well as the light-petroleum extracts of the segments, were combined in such a way as to correspond to the three radioactive peaks separated by electrophoresis, namely the peaks at the point of application, at the α_2-globulin and at the albumin. These fractions were split into two portions: 40 % for radioautography as described above, and 60 % for biological assay.

(*c*) Biological assay. The vitamin D activity present in the various extracts was assayed by using the method of Bourdillon, Bruce, Fischmann & Webster (1931). The rachitic rats given the test doses were X-rayed 2 weeks later, and the healing was compared with that obtained in rats dosed with 40, 20 and 10 i.u. of vitamin D_3. The extracts from Expt. 1 were combined into the α_2-globulin and albumin peaks, as described above for Expt. 2. As no radioactive material was found at the origin, this region was not assayed for vitamin D activity. In view of the small amount of vitamin D present, only a one-point assay was possible, with two to five rats. The doses were fed on squares of rice-paper.

RESULTS

Protein analysis after starch electrophoresis

Four clear peaks were found: a fast-moving, large albumin fraction, with the α_1-globulin appearing as a shoulder, followed by small α_2-, β- and γ-globulin peaks (Figs. 1, 2). The albumin peak moved further in Expt. 1 than in Expt. 2, giving a better separation.

Distribution of radioactive material after electrophoresis

Expt. 1. Two radioactive peaks were found in the distribution of both the saline and light-petroleum extracts. One corresponded with α_2-globulin and the other with albumin (Fig. 1). Table 1 shows the quantitative distribution of radioactive material between the two proteins. It differed in the two methods of extraction. Light petroleum extracted 17·5 % of the radioactive material from the α_2-globulin and 30·5 % from the albumin fraction; saline extracted 10·5 % from α_2-globulin and 10·1 % from albumin. Hence light petroleum extracted

Table 1. *Relationship between extracted* ^{14}C*-labelled material and the* α_2*-globulin and albumin fractions (Expt. 1)*

	Recovery of added ^{14}C (%)			^{14}C-Labelled material per unit protein	
	In saline (*A*)	In light petroleum (*B*)	Protein (*C*)	In saline (*A*/*C*)	In light petroleum (*B*/*C*)
α_2-Globulin fraction	10·5	17·5	17	0·62	1·03
Albumin fraction	10·1	30·5	57	0·18	0·54
Total	20·6	48·0	—	—	—

Table 2. *Relationship between extracted* ^{14}C*-labelled material and the protein fractions (Expt. 2)*

	Recovery of added ^{14}C (%)				^{14}C-Labelled material per unit protein		
	In saline (*A*)	In light petroleum (*B*)	In ethanol–diethyl ether (*C*)	Protein (%) (*D*)	In saline (*A*/*D*)	In light petroleum (*B*/*D*)	In ethanol–diethyl ether (*C*/*D*)
α_2-Globulin fraction	24·5	28·5	39·2	19	1·29	1·50	2·06
Albumin fraction	14·2	21·1	33·9	61	0·23	0·35	0·56
Origin	9·9	13·1	18·2	0	—	—	—
Total	48·6	62·7	91·3				

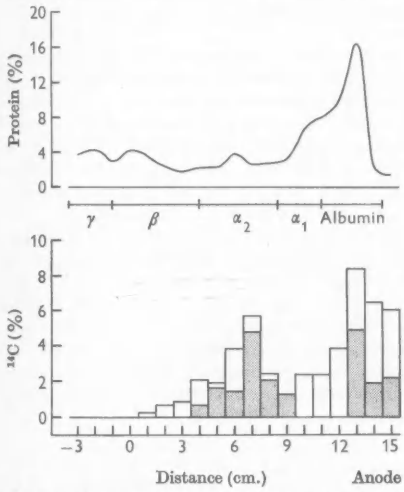

Fig. 1. Distribution of ^{14}C after electrophoresis of rat serum incubated with [^{14}C]vitamin D_2 (Expt. 1). ▤, ^{14}C extractable with saline; □, excess of ^{14}C extractable with light petroleum; α_1, α_2, β and γ refer to the respective globulins.

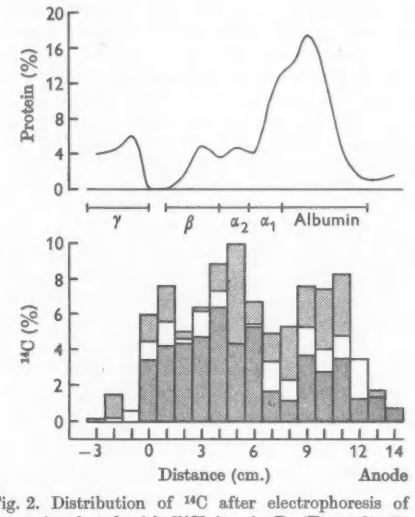

Fig. 2. Distribution of ^{14}C after electrophoresis of rat serum incubated with [^{14}C]vitamin D_2 (Expt. 2). ▤, ^{14}C extractable with saline; □, excess of ^{14}C extractable with light petroleum; ▨, residual ^{14}C extractable, after boiling with methanol, with ethanol–diethyl ether; α_1, α_2, β and γ refer to the respective globulins.

48·0 %, and saline extracted 20·6 %, of the added radioactive material.

The ^{14}C-labelled material per unit protein was calculated from the ratio of recovered ^{14}C to the amount of protein. In the light-petroleum extracts, the α_2-globulin fraction showed a higher ratio (1·03) than the albumin fraction (0·54), although the albumin carried more total radioactive material. In the saline extracts, again, the corresponding ratios to protein were greater in the α_2-globulin (0·62) than in the albumin fraction (0·18).

Expt. 2. A study was made of the distribution of radioactive material extractable by saline followed by light petroleum, and then by more drastic

extraction techniques. All three procedures showed the two ^{14}C-labelled peaks observed in Expt. 1 and in addition a third peak at the point of application (Fig. 2). In Table 2, the first three columns show the calculated percentage of radioactive material extractable by each procedure. Since the different extractions were performed successively, and only a portion of the saline extract was used for analysis of ^{14}C, correction was made for this procedure. It was shown (see Recovery of radioactive material) that light petroleum extracted the portion also extractable by saline; the ethanol–diethyl ether procedure extracted all or almost all the ^{14}C-labelled material. The data quoted for the ethanol–diethyl ether procedure therefore includes the material extracted by light petroleum, and that given for the light-petroleum extraction includes the material removed by saline.

Saline thus extracted 48·6 %, light petroleum 62·7 % and ethanol–diethyl ether (after boiling with methanol) 91·3 %. The recoveries were greater than in Expt. 1, particularly because of the amount found in the α_2-globulin fraction. The total radioactive material as obtained by ethanol–diethyl ether extraction was 39·2 % from the α_2-globulin, 33·9 % for the albumin fraction and 18·2 % from the origin. As in Expt. 1, the ^{14}C-labelled material per unit protein was several times greater in the α_2-globulin fraction than in the albumin, irrespective of the method of extraction.

Recovery of radioactive material

Preliminary experiments to test the extraction procedures were done by measuring the total radioactive material recoverable direct from serum or from serum mixed with starch without recourse to electrophoresis. For each determination 1 ml. of serum containing 2·5 μg. of ^{14}C-labelled vitamin D_2 was used. The different extraction procedures used to recover vitamin D were investigated: extraction with saline (procedure 1), light petroleum (procedure 2) and ethanol–diethyl ether after refluxing with methanol (procedure 3). Extracts were made of ^{14}C-labelled vitamin D_2 incubated with serum, of the same left overnight on a 5 g. starch block at 7°, and of ^{14}C-labelled vitamin D_2 in propylene glycol or ethanol, without serum, left on a starch block overnight.

The recoveries are shown in Table 3. Light petroleum extracted 86 and 91 % of the radioactive material from starch in the absence of serum. In the presence of serum the percentage extractable was reduced to 50 %. Saline extracted 46 and 37 % of the radioactive material in the absence of serum, and 25 % in the presence of serum. On refluxing with methanol, followed by extraction with ethanol–diethyl ether, a recovery of 95 % of the radioactive material was obtained even in the presence of serum.

Identity of radioactive material with vitamin D

Both biological assay and reversed-phase paper chromatography showed that only a fraction of the radioactive material recovered was vitamin D (Table 4).

The reversed-phase paper chromatography permitted the quantitative determination of the radioactive material from the control experiments, mentioned above, whereas the ^{14}C counts on paper chromatograms for the Expts. 1 and 2 only allowed a qualitative picture (Fig. 3). Biological results were obtained for the various fractions in Expts. 1 and 2 as shown in Table 4.

After incubation of vitamin D with serum, only 27 % of the radioactive material recoverable with light petroleum was still the vitamin. The same results were obtained after leaving the incubated vitamin D on starch overnight. The recoveries of vitamin D in the radioactive material that was obtained from the origin, α_2-globulin and albumin fractions by saline or by light-petroleum extraction, ranged from 10 to 38 %.

Table 3. *Recovery of* ^{14}C*-labelled material*

Sample	Recovery of added ^{14}C-labelled material (%)
1. Saline extract of	
^{14}C-Labelled vitamin D_2 incubated with serum, left on starch overnight	25
^{14}C-Labelled vitamin D_2 in propylene glycol left on starch overnight	46
^{14}C-Labelled vitamin D_2 in ethanol left on starch overnight	37
2. Light-petroleum extract of	
^{14}C-Labelled vitamin D_2 incubated with serum	50
^{14}C-Labelled vitamin D_2 incubated with serum, left on starch overnight	50
^{14}C-Labelled vitamin D_2 in propylene glycol left on starch overnight	86
^{14}C-Labelled vitamin D_2 in ethanol left on starch overnight	91
3. Ethanol–diethyl ether extract, after methanol treatment	
^{14}C-Labelled vitamin D_2 incubated with serum, left on starch overnight	95
^{14}C-Labelled vitamin D_2 in propylene glycol left on starch overnight	98

Table 4. *Identity of radioactive material with vitamin D*

Control experiments	Vitamin D: From ^{14}C count of sample ($\mu g.$)	Vitamin D: By biological assay ($\mu g.$)	Vitamin D: By biological assay (% of radioactive material)	Vitamin D: By paper chromatography* (% of radioactive material)
Light-petroleum extract of				
^{14}C-Labelled vitamin D_2 + serum	5·0	—	—	27
^{14}C-Labelled vitamin D_2 + serum on starch	5·0	—	—	28
Expt. 1				
Saline extract of				
α_2-Globulin fraction	1·05	0·10	10	—
Albumin fraction	1·00	0·25	25	—
Light-petroleum extract of				
α_2-Globulin fraction	0·65	0·16	25	—
Albumin fraction	2·05	0·26	13	—
Expt. 2				
Saline extract of				
α_2-Globulin fraction	1·2	0·25	21	—
Albumin fraction	0·7	0·17	24	—
Origin	0·5	0·15	29	—
Light-petroleum extract of				
α_2-Globulin fraction	1·6	0·46	29	—
Albumin fraction	1·4	0·58	38	—
Origin	0·8	0·28	35	—

* The percentage count was obtained by calculating the ratio of radioactivity of the vitamin D spot to that of the whole paper strip.

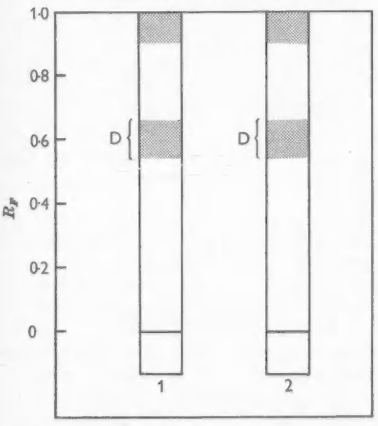

Fig. 3. Radioautographs of reversed-phase paper chromatograms of light-petroleum extracts. 1, Extract of ^{14}C-labelled vitamin D_2 incubated with serum, left on starch; 2, extract of a 1 cm. segment from Expt. 1 (13 cm. from the point of application); D, area corresponding to that occupied by the unlabelled vitamin D marker.

DISCUSSION

In the absence of serum, vitamin D did not move from the origin, as shown by radioautography of papers after electrophoresis of the pure vitamin. After incubation with rat serum, vitamin D acquired electrophoretic mobility, suggesting an interaction between serum and vitamin D. The proteins involved were α_2-globulin and albumin. Although the separation of α_1-globulin from albumin was not good, the position of the vitamin D seemed to correspond with the albumin peak. In Expt. 2 the third peak at the origin, consisting of about one-fifth of the ^{14}C-labelled material, might be excess of vitamin D, owing to the high levels used in these experiments. Previously, with ten times the concentration of vitamin D, almost all was found at the origin.

The distribution of radioactive material between the two proteins was approximately equal, the albumin carrying a little more in the first experiment and the α_2-globulin in the second. Owing to the high concentration of albumin in serum, the capacity of α_2-globulin for vitamin D, in terms of radioactive material per unit protein, was about two to four times as great as that of albumin. The distribution of vitamin D in rat serum confirms the results obtained by the Baltimore workers (Morgan *et al.* 1958; Thomas *et al.* 1959) with human serum, in which they used unlabelled vitamin and assayed it biologically. The use of ^{14}C-labelled vitamin D and the modified technique made it possible to obtain the vitamin content at 1 cm. intervals without pooling the samples. The greater resolution obtained by this method gave two separate

fractions of vitamin D, thereby showing an association with both α_2-globulin and albumin. Using the difference in extractability by light petroleum and ethanol–diethyl ether of the free and protein-bound vitamin D, Kodicek, Cruickshank & Ashby (1960) confirmed that the main portion of ^{14}C-labelled vitamin D_2 in the blood of rats injected intracardially with the vitamin is bound to protein.

It thus appears that vitamin D reaching the blood stream, either from the intestinal lumen or direct, is transported by the plasma proteins.

The complete recovery of biologically-active vitamin D obtained by Morgan *et al.* (1958) was not repeatable in our experiments. Both biological assay and reversed-phase paper chromatography showed that destruction of the vitamin had occurred during the experiment, about three-quarters being rendered inactive. These findings were not related to the type of extraction procedure employed; such destruction was found even in the light-petroleum extracts of unfractionated serum. As leaving the vitamin, incubated with serum, on starch overnight had no deleterious effect, it is assumed that this was not the cause of destruction. Also there was no more destruction during electrophoresis. For vitamin A a protection by the serum proteins during electrophoresis was claimed by Erwin *et al.* (1959). It appears thus that destruction of the vitamin D may have occurred during incubation or during extraction.

Attempts were made to determine by differential-extraction procedures the type and strength of binding. It was hoped that saline would extract the water-soluble material and give a fraction containing protein and tightly bound vitamin D. However, saline extracted about one-half of the vitamin even in the absence of serum. This could be explained by dispersion of the vitamin between starch and saline. More informative results were obtained from the fraction extractable with light petroleum. Although nearly all the vitamin D could be extracted with light petroleum in the absence of serum, in its presence only half was extractable, indicating the presence of a protein–vitamin D complex. The residue could be recovered by ethanol–diethyl ether extraction after splitting the protein bonding with methanol. These results *in vitro* were confirmed by experiments *in vivo* with rats (Kodicek, Cruickshank & Ashby, 1960) and with infants (Kodicek, Cruickshank & Stapleton, 1960).

If vitamin D were carried by cholesterol (or another lipid) in the lipoprotein fractions, a correlation between its distribution and that of cholesterol would be expected. The distribution of cholesterol in serum (Kunkel & Slater, 1952*a*, *b*; Boyd, 1954) does not coincide with that found by us for vitamin D, and therefore it appears unlikely that the lipoproteins are concerned with the transport of the vitamin.

SUMMARY

1. The distribution of ^{14}C-labelled vitamin D_2 in serum proteins has been studied. The vitamin was incubated in high physiological concentration (2·5 μg./ml. of serum) in rat serum, and the radioactive material separated by electrophoresis on a starch-supporting medium. Segments of the starch block were eluted with 0·9% sodium chloride solution, with light petroleum and, after refluxing with methanol, with ethanol–diethyl ether.

2. The radioactive material was found to correspond with the α_2-globulin and albumin peaks. Although similar amounts were extracted from the two proteins, the α_2-globulin fraction had a bigger capacity per unit protein than the albumin fraction.

3. In some instances, the remaining radioactive material did not move from the point of application, showing that it was not associated with electrophoretically mobile components. This was probably because the vitamin was present in excess of the binding capacity of the proteins.

4. Biological assay and reversed-phase paper chromatography showed that only about one-quarter of the radioactive material recovered was vitamin D. It seemed probable that breakdown occurred during incubation or extraction.

5. In the presence of serum, light petroleum extracted only about one-half of the total vitamin D. The residue could be recovered by splitting the protein bonding with boiling methanol.

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Carbohydrase and Sulphatase Activities of *Porphyra umbilicalis*

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The carbohydrate constituents of the red seaweeds (Rhodophyceae) are characterized by the frequent occurrence of galactose, its derivatives and polymers. The mucilaginous substance of the algae, often present in very large quantities (up to 80% of the dry weight), is usually a polysaccharide composed of residues of D-galactose and certain of its derivatives, such as galactose sulphate, 3:6-anhydrogalactose, 6-*O*-methylgalactose, as well as L-galactose. Sucrose, a key substance in higher plants, is absent here and its functions are probably taken over by floridoside (Bean & Hassid, 1955; Bidwell, 1958), which is a glycerol galactoside (2-*O*-α-D-galactopyranosylglycerol), commonly found in the red seaweeds. Compounds related to floridoside have also been reported from some members of this class. Such compounds are 1-*O*-α-D-galactopyranosyl-D-glycerol and 1-*O*-α-D-galactopyranosyl-L-glycerol from *Porphyra umbilicalis* (Lindberg, 1955*b*; Wickberg, 1958*b*); 2-*O*-α-D-mannopyranosyl-D-glyceric acid from *Polysiphonia lanosa* and *Ceramium rubrum* (Colin & Augier, 1939; Bouveng, Lindberg & Wickberg, 1955); *O*-α-D-galactopyranosyl-(1 → 6)-*O*-β-D-galactopyranosyl-(1 → 1)-D-glycerol from *Polysiphonia lanosa* and *Corallina officinalis* (Wickberg, 1958*a*); and *O*-α-D-mannopyranosyl-(1 → 3)-*O*-α-D-galactopyranosyl-(1 → 2)-glycerol from *Furcellaria fastigiata* (Lindberg, 1955*a*).

A common reserve polysaccharide of the red seaweeds is floridean starch, which contains no amylose component and is a polysaccharide of the glycogen–amylopectin type (Fleming, Hirst & Manners, 1956; Peat, Turvey & Evans, 1959).

The synthesis and transformation of carbohydrates in these seaweeds has as yet received little attention. Some of the enzyme activities present in extracts of *Rhodymenia palmata* and *Iridophycus flaccidum* have been described (Duncan, Manners & Ross, 1956; Bean & Hassid, 1956), and the fate of $^{14}CO_2$ after incorporation into certain species has also been studied (Bean & Hassid, 1955; Bidwell, 1958).

Seaweeds of the genus *Porphyra* belong to this class. *Porphyra umbilicalis* grows on the Welsh coast and is collected and marketed in quantity for human consumption. It is a relatively primitive organism, belonging to the subclass Bangioideae (Fritsch, 1945). The carbohydrates present have been investigated by a number of workers. The major polysaccharide (which sometimes represents over 30% of the dry weight), to which the name of porphyran is given, contains residues of D-galactose, L-galactose, 3:6-anhydro-L-galactose, 6-*O*-methyl-D-galactose and galactose ester sulphate (Turvey & Rees, 1958), much of the ester sulphate being carried at the 6-position of L-galactose residues (Turvey & Rees, 1961). A similar polysaccharide has been isolated from *Porphyra capensis* (Nunn & von Holdt, 1957). The presence of floridean starch has been demonstrated in *Porphyra umbilicalis* (Turvey & Rees, 1958), and the principal low-molecular-weight carbohydrates have been shown to be floridoside and isofloridoside (Lindberg, 1955*b*). The latter is stated to be a mixture of 1-*O*-α-D-galactopyranosyl-D-glycerol and 1-*O*-α-D-galactopyranosyl-L-glycerol (Wickberg, 1958*b*). *Porphyra* is unusual in the nature of the skeletal polysaccharide of the cell wall, which is not cellulose but a β-1:4-linked polymannose (Jones, 1950; Cronshaw, Myers & Preston, 1958).

The purpose of this paper is to describe the results of a preliminary survey of the enzymes present in extracts of *Porphyra umbilicalis*, particular

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attention being paid to the enzyme-catalysed reactions involving derivatives or polymers of galactose.

EXPERIMENTAL

Materials

Substrates. The preparation and characterization of porphyran and floridean starch are described in a separate communication (Peat, Turvey & Rees, 1961). Analysis of porphyran gave the values shown in Table 1. It showed $[\alpha]_D$ −79°. Oligosaccharide A is a mixture of related substances with a degree of polymerization (D.P.) of approximately 20, precipitated by the addition of 2–3 vol. of ethanol to a partial hydrolysate of porphyran. Oligosaccharide B was obtained similarly by the addition of a further 8 vol. and has D.P. 5–7. The conditions used for the partial hydrolysis (0·5 N-sulphuric acid at 50° for 10 hr.) were such that no sulphate was released from ester linkages, although the polysaccharide was extensively depolymerized. The two fractions, oligosaccharide A and oligosaccharide B, were the main sulphated components isolated, and contained between them most of the esterified sulphuric acid originally present. The floridean starch used in these experiments was contaminated with galactan. Glucose 6-sulphate and glucose 3-sulphate were prepared in this laboratory by Mr T. P. Williams, galactose 6-sulphate by Dr K. O. Lloyd, nigerose by Dr J. Mon Evans, and the cellodextrins by Dr J. G. Roberts by precipitation from a partial hydrolysate of cellulose, by the addition of ethanol to a concentration of 20%. Yeast mannan was prepared by Dr D. Doyle (1959); the dextran was supplied by Dextran Ltd., Aycliffe, Darlington, Co. Durham (see Turvey & Whelan, 1957), and inulin by Thomas Kerfoot Ltd., Vale of Bardsley, Lancs.

Seaweed. Samples of *Porphyra umbilicalis* (L.) Kutz. f. *umbilicalis* (Parke, 1953) were gathered, except where otherwise stated, at Aberdesach near Caernarvon on the North Wales coast, in the period November 1959 to May 1960.

Methods

Unless otherwise stated, the evaporation of all solutions was carried out under diminished pressure, at pH 5–7 and at 35–40°.

Incubations of enzymes with substrates were carried out in a thermostat at 35° in the presence of a preservative (usually toluene).

Paper chromatography. This was effected by downward development with the following solvent systems (proportions by volume): (i) butanol–pyridine–water (6:4:3); (ii) butanol–ethanol–water (5:1:4); (iii) butanol–acetic acid–water (4:1:5); (iv) propanol–ethyl acetate–water (7:1:2). Sugar zones were located with alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950), *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950) or periodate–benzidine (Cifonelli & Smith, 1954). Whatman no. 1 paper was normally used.

Table 1. *Composition of porphyran (sodium salt)*

	Percentage*	Molar ratio
D-Galactose	16·0	1·00
L-Galactose	17·6	1·10
6-*O*-Methyl-D-galactose	20·6	1·18
3:6-Anhydro-L-galactose	10·3	0·73
Esterified sulphate	11·1	1·40
Nitrogenous material (calc. as protein)	1·8	—
Associated inorganic material other than esterified sulphate	13·9	—
Other sugar residues	0·8	—

* Sugars are calculated as anhydro units.

Viscosity measurements. These were performed in an Ostwald viscometer with a flow time of 10·7 sec. for water (4 ml.). Experiments were carried out in a thermostat at 35°.

Reducing power. This was determined with the Somogyi (1945*a*) reagents after deproteinization (Somogyi, 1945*b*), or with the Shaffer & Hartmann (1921) reagent. With the latter, deproteinization was unnecessary with 1 ml. samples of mixtures containing 0·5% (w/v) of protein.

Detection of sulphatase activity. The incubation mixture (1·5 ml.) was treated with trichloroacetic acid (40%, w/v, 0·1 ml.); the precipitated protein was coagulated by warming in a water bath at 70–85° and removed on the centrifuge. A portion of the supernatant solution (1 ml.) was withdrawn and tested with acidic barium chloride [2% (w/v) barium chloride in 0·1 N-hydrochloric acid]. The presence of liberated sulphate ions was indicated by the development of a turbidity within 5 min.

The desulphation of monosaccharide derivatives was detected by the appearance of the free monosaccharide on a paper chromatogram.

Isolation of low-molecular-weight carbohydrates of the seaweed

The method was similar to that of Lindberg (1955*b*). Pressed *Porphyra umbilicalis* (7 kg. wet wt.), collected in October 1959, was minced into ethanol (9 l.) soon after harvesting. The mixture was brought to the boil, then left for 24 hr., and filtered successively through a nylon stocking, a pad of Celite and a pad of charcoal (British Drug Houses Ltd.; 'activated'). The solution was adjusted to pH 6, and carefully evaporated to 1 l. The precipitate was removed on the centrifuge, the solution de-ionized by treatment with Zeo-Karb 225 (H^+) and De-Acidite FF (CO_3^{2-}) and evaporated to a brown immobile syrup (125 g.). Part of this product (8·5 g.) was adsorbed on a charcoal–Celite column (130 cm. × 5 cm.; equal parts by weight of 'activated' charcoal and Celite). The column was eluted with aqueous ethanol, the composition of which was changed continuously in a linear manner from 1·5 to 16·5% (v/v) over a total volume of 20 l. The column was finally eluted with aq. 10% (v/v) butan-2-one. Fractions (150 ml. each) were collected and analysed for carbohydrate by using the phenol–sulphuric acid reagent (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Two major peaks were observed but these were incompletely separated. The fractions containing carbohydrate were therefore examined by paper chromatography, and those containing a mixture of two substances as indicated by this method were rejected. The remaining fractions were combined to give two solutions, each containing a chromatographically pure component. These solutions were each evaporated to dryness and the residues crystallized from ethanol.

Identification of floridoside. The slower-moving substance on charcoal, and the faster-moving on paper [rate of flow relative to galactose ($R_{Gal.}$) in solvent (ii) on Whatman no. 3

paper, was 1·10] was non-reducing and on acid hydrolysis gave galactose and glycerol as indicated by paper chromatography. Weight 0·58 g.; m.p. 127–128·5° (capillary, uncorr.); $[\alpha]_D$ +163° in water (*c*, 0·36). These properties are in good agreement with those previously reported for floridoside (Lindberg, 1955*a*, *b*, Putman & Hassid, 1954; Nunn & von Holdt, 1955).

Identification of isofloridoside. The faster-moving substance on charcoal and the slower-moving on paper ($R_{Gal.}$, with the same conditions as above, was 0·90), resembled the first component in that it was non-reducing and on acid hydrolysis it gave galactose and glycerol as indicated by paper chromatography. Weight 1·50 g.; m.p. 149·5–151·5°. A sample of isofloridoside was melted on the block of the Kofler apparatus, allowed to cool, seeded and left to crystallize, and the m.p. was retaken (found 147–150°), $[\alpha]_D$ +155°. The chromatographic behaviour and the specific rotation were as expected for isofloridoside (Lindberg, 1955*b*; Wickberg, 1958*b*).

Isolation and examination of isofloridoside from another sample of Porphyra. This sample of *Porphyra* was collected by Dr E. Conway from the splash zone on the island of Colonsay, Scotland, in September 1959. The air-dried seaweed (10·5 g.) was cut into small pieces and extracted briefly with boiling water (4 × 100 ml.). Polysaccharide was precipitated by the addition of ethanol (4 vol.) and removed on the centrifuge. The solution was evaporated to dryness, the residue redissolved in water and the resulting solution de-ionized [Zeo-Karb 225 (H^+) and De-Acidite FF (CO_3^{2-})] and concentrated to a small volume. The isofloridoside was separated from the mixture by thick-paper chromatography [multiple development on Whatman no. 3 paper and solvent (ii)], dissolved in ethanol, decolorized with charcoal and crystallized from ethanol. Weight 0·059 g.; m.p. 148·5–151·5° (Kofler hot-stage, corr.).

Extraction of the enzymes

Fresh pressed seaweed (5 kg.) was minced (Kenwood mixer) into aq. 0·25% sodium carbonate (7–8 l.) precooled to 4°. The mixture was left at this temperature overnight, pressed through muslin and clarified on the centrifuge. To the resulting solution (pH 8·2) was added solid ammonium sulphate to 0·8 saturation; the precipitate was removed on the centrifuge, dissolved in water and the solution dialysed against running tap water at 5–10° until a negative test for sulphate was obtained (the test being that described above for the detection of sulphatase activity). The dialysis bag was changed daily, otherwise it became so weakened that it burst after 2–4 days. This phenomenon was probably due to the presence of a cellulase. The sediment which appeared during dialysis was removed on the centrifuge and the clear orange to red supernatant solution was freeze-dried, giving a red to purple powder (about 5 g.). Several other extraction procedures were used in early investigations, but this method was preferred because it gave the greatest yield of sulphatase activity.

RESULTS

Carbohydrase activity. The freeze-dried extract (5 mg.) was incubated in potassium acetate buffer (0·05 M; pH 6·5; 1 ml.) with each of a number of carbohydrates (5 mg.), and the extent of the hydrolysis of the substrates was followed by paper chromatography. The summarized results are given in Table 2, in which 'marked hydrolysis' means the release of appreciable quantities of hydrolysis products within 48 hr.

Transgalactosylation activity. When the freeze-dried extract (10 mg.) was incubated with floridoside (150 mg.) in potassium acetate buffer (0·1 M; pH 6·5; 1 ml.) for periods up to 3 weeks, there was only doubtful evidence for the formation of a higher saccharide [$R_{Gal.}$ in solvent (i) 0·28?]. It is therefore concluded that if transgalactosylation reactions are catalysed by the α-galactosidase, the activity is very weak when floridoside is the substrate.

Sulphatase activity. When the freeze-dried extract [1% (w/v) in 0·1 M-potassium acetate buffer, pH 6·5; 0·5 ml.] was incubated with porphyran [1% (w/v) in water; 1 ml.], free sulphate

Table 2. *Enzyme activities found in extracts of* Porphyra umbilicalis

Type of activity detected	Marked hydrolysis of substrate	Slight hydrolysis of substrate	Weak, doubtful or no hydrolysis of substrate
α-Galactosidase	Floridoside	Isofloridoside, melibiose	Raffinose, α-methyl galactoside
β-Galactosidase	—	Lactose	β-Methyl galactoside
α-Glucosidase	Sucrose, maltose, nigerose	α-Methyl glucoside	—
β-Glucosidase	—	Cellobiose, cellodextrins	—
Amylase	Amylose, amylopectin, glycogen, floridean starch	—	—
β-Glucanase	Laminarin, cellophan	—	—
Mannanase	Ivory-nut mannan	—	Yeast mannan
Miscellaneous polysaccharases	—	—	Dextran, inulin
Sulphatase	Porphyran	—	Oligosaccharide A, oligosaccharide B, galactose 6-sulphate, galactose disulphate, glucose 3-sulphate, glucose 6-sulphate

was found in the solution within 3 hr. Certain other carbohydrate sulphates, however, were hydrolysed very much less readily (Table 2).

Other enzymes acting on porphyran. The incubation mixture of the enzyme extract and porphyran showed no increase in reducing power after 48 hr., and no low-molecular-weight hydrolysis products could be detected in it by paper chromatography, even after prolonged periods. The only change in the polysaccharide which could be detected concomitant with sulphate release was a fall in viscosity. This fall in viscosity was shown to be due to an enzyme other than the sulphatase by the following experiment. Two digests were prepared by mixing porphyran [1 % (w/v) in water; 3 ml.] with freeze-dried extract [1 % (w/v) in 0·1 M-buffer, pH 6·5; 1·5 ml.]. One of the digests contained sodium citrate buffer and the other potassium acetate. Inhibition of the sulphatase occurred in the mixture containing citrate (a barely detectable opalescence was observed after 48 hr.), whereas a relatively large amount of sulphate was released in the other (an almost opaque turbidity in 24 hr.). In contrast, the two digests showed similar decreases in viscosity (acetate-containing digest showed $\eta_{sp.}$ 1·06 → 0·51 in 16 hr., citrate-containing digest, $\eta_{sp.}$ 1·06 → 0·58). In control experiments, neither the polysaccharide nor the extract separately showed any change in viscosity, even when incubated for prolonged periods.

DISCUSSION

The low-molecular-weight carbohydrates were investigated since they were possible natural substrates for the enzyme system. The two major components of this group have properties similar to those of the glycosides isolated by Lindberg (1955*b*) from a *Porphyra umbilicalis* of different origin. One component was in fact proved to be floridoside (2-*O*-α-D-galactopyranosylglycerol), but the properties of our second component were not entirely consistent with the description given by Wickberg (1958*b*) of Lindberg's sample of isofloridoside. The substance was non-reducing, gave galactose and glycerol on hydrolysis, the specific rotation was close to that quoted for isofloridoside, and its behaviour, when chromatographed on charcoal, was similar to that described by Lindberg. According to Wickberg, however, isofloridoside is a mixture of two diastereomers, 1-*O*-α-D-galactopyranosyl-D-glycerol and 1-*O*-α-D-galactopyranosyl-L-glycerol, which are isomorphous and give no melting-point depression when mechanically mixed, but which melt over a very wide range (130–150°), corresponding to the difference between the melting points of the two components (131·5–133° and 149–151°), although a low-melting zone (120°) was observed when the substance was examined by the mixed fusion technique. We have, however, been unable to detect any product melting below 149·5° in the sample isolated in the present study. Moreover, the melting point of our isofloridoside was virtually unchanged after crystallization from the molten state (147–150°).

It would seem, therefore, that this specimen of isofloridoside consists only of 1-*O*-α-D-galactopyranosyl-D-glycerol, that is, of the isomer with the higher melting point (Wickberg, 1958*b*). The values for the specific rotations of the diastereomers are too close to permit any conclusions about homogeneity to be drawn from the measured value. The infrared-absorption curves obtained by Wickberg for the two synthetic diastereomers showed that this technique was not suitable to determine whether small quantities of the L-glycerol derivative were present in our preparation.

It is difficult to account for the disparity of Wickberg's and our results in regard to the structure of isofloridoside. It is not possible that the missing diastereomer was present in the discarded charcoal chromatography fractions, because the L-glycerol derivative has the greater mobility (Wickberg, 1958*b*), and only the slower-moving fractions were rejected. The disparity may be due to differences between the seaweed sources used. Some confusion still surrounds the botanical classification of the genus *Porphyra*, and it is possible that the seaweed examined by the Swedish workers differs ecologically or seasonally from that taken for the present study (Dr E. Conway, personal communication). The sample which we examined originally was gathered low down in the intertidal belt of a shore that is comparatively sheltered. A second sample was therefore examined, taken from a very exposed coast where the plant is found above the high-tide level so that it is probably under conditions much less favourable for metabolism and growth. However, the melting-point characteristics of the isofloridoside thus isolated again gave no indication of the presence of a second component.

Although floridoside is present in a large number of red algae, isofloridoside has been reported as present only in *Porphyra umbilicalis*, and Lindberg has suggested that it may be peculiar to the subclass Bangioideae. It is reasonable to suppose that floridoside performs the same biological function in the Bangioideae as in the Florideae, namely that it provides a food reserve. The *Porphyra* extracts showed a marked hydrolytic activity towards floridoside, but a much less pronounced activity on isofloridoside and other α-galactosides (Table 2). The only observation that can be made at this stage concerning the role of isofloridoside is that if the α-galactosidase is responsible for the break-

down of floridoside *in vivo*, then isofloridoside cannot be regarded as interchangeable with the floridoside as a reserve substance, although both are α-glyceryl galactosides. It is tempting to suggest that the floridoside–α-galactosidase system here is analogous to the sucrose–invertase system of higher plants. An important difference, however, is that, whereas invertase shows a fairly marked ability to catalyse transglycosylation reactions (Allen & Bacon, 1956), such reactions occur in the α-galactosidase system only to a slight extent, if at all. In accordance with this view is the fact that some of the oligosaccharides which are formed by the mediation of invertase *in vitro* are often found associated with sucrose *in vivo* (cf. Pridham, 1960), but in contrast no oligosaccharides have yet been reported as present in the red seaweeds which could have arisen by trans-α-galactosylation with floridoside as acceptor. In the study of the low-molecular-weight carbohydrates of *Porphyra* reported in this paper, there is indeed no evidence for the presence of any oligosaccharide material at all. There is not yet sufficient information available to enable a useful comparison to be made between the α-galactosidase of *Porphyra* and similar enzymes from other sources (cf. Gottschalk, 1958; French, 1954).

The other carbohydrases of *Porphyra umbilicalis* were as anticipated on the basis of the survey made by Duncan *et al.* (1956) of the carbohydrase activities of extracts of *Cladophora rupestris*, *Ulva lactuca*, *Laminaria digitata* and *Rhodymenia palmata*, with the difference that the β-glucosidase activity towards cellobiose and cellodextrins was much weaker in the *Porphyra* extracts. It should be noted that *Porphyra*, apparently alone amongst seaweeds, is devoid of cellulose (Cronshaw *et al.* 1958). There is thus further reason to believe that the enzyme pattern of α- and β-glucosidase, amylase, β-1:3- and β-1:4-glucanase and mannanase activities found by Duncan *et al.* is a general one present in most seaweeds.

The pronounced carbohydrate-sulphatase activity which was detected in the extracts is of interest because of the occurrence of very substantial deposits of galactan sulphate in the intercellular regions of living *Porphyra*. Although this is the first recorded instance of the extraction of an enzyme of this type from a seaweed, there have been repeated reports of sulphatases from various other marine organisms (for references see Dodgson & Spencer, 1956), and K. S. Dodgson (personal communication) has detected the liberation of sulphate in seaweed extracts, presumably from the interaction of an enzyme with a coextracted substrate.

The chondrosulphatase of *Proteus vulgaris* (Dodgson & Lloyd, 1957) has been shown to require extensive depolymerization of the polysaccharide before desulphation can occur, i.e. the true substrate of this enzyme is not chondroitin sulphate but the sulphated oligosaccharides produced from it by the action of the associated chondroitinase. The sulphatase present in *Porphyra* extracts does not appear to function in this way, its substrate being undegraded porphyran. This follows because there is no increase in the reducing power of the polysaccharide concomitant with desulphation, and there is only a very slight activity exhibited by the enzyme towards low-molecular-weight carbohydrate sulphates, including certain oligosaccharide sulphates derived from porphyran (Table 2).

One qualification attaches, however, to this interpretation. By inhibition of the sulphatase with citrate it was shown that another enzyme having action on porphyran is present in the crude preparation. The only information at present available concerning the mode of action of this enzyme is that it produces a fall in the viscosity of the polysaccharide when incubated with it. Nevertheless, it is quite possible that, even though no low-molecular-weight fragments are produced (since none were detected on paper chromatography, and since there was no increase in reducing power), this activity is of the carbohydrase type. It might, for example, have a debranching action resulting in the production of high-molecular-weight dextrins, which have a negligible copper-reducing power. The conclusion is reached, therefore, that extensive depolymerization is not a necessary preliminary to desulphation of porphyran by enzymes present in *Porphyra* extracts, although fragmentation of the macromolecule into units which are still relatively large may be required. With respect to its molecular-weight specificity, this enzyme thus resembles the cellulose polysulphatase found in liver extracts of a marine mollusc by Takahashi & Egami (1960) rather than the chondrosulphatase of *Proteus vulgaris*.

SUMMARY

1. A qualitative investigation has been made of some enzymes present in extracts of the red seaweed *Porphyra umbilicalis*, particular attention being paid to those which catalyse reactions involving derivatives and polymers of galactose.

2. The principal low-molecular-weight carbohydrates of the alga have been isolated since they were potential substrates for the enzyme system. These were floridoside and isofloridoside. It is concluded that in the seaweed samples examined in the present study, the isofloridoside component is 1-*O*-α-D-galactopyranosyl-D-glycerol.

3. *Porphyra umbilicalis* extracts exhibit many of the carbohydrase activities previously reported

for other algal extracts, namely α- and β-glucosidase, amylase, β-glucanase and mannanase.

4. An α-galactosidase activity is also present for which floridoside is possibly the natural substrate. Some implications of this, in terms of the carbohydrate metabolism of the seaweed, are discussed.

5. At least two enzymes having action on porphyran, the principal component of the plant, are present. One of these is a sulphatase which is inhibited by citrate, and the other catalyses a reaction causing a fall in the viscosity of the polysaccharide solution.

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Equilibrium Constants of Phosphoryl Transfer from $C_{(1)}$ to $C_{(6)}$ of α-D-Glucose 1-Phosphate and from Glucose 6-Phosphate to Water

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The calculation of the free energy of hydrolysis of adenosine triphosphate (Atkinson, Johnson & Morton, 1959; Atkinson & Morton, 1960) from the equilibrium constant of the reaction catalysed by galactokinase (Atkinson, Burton & Morton, 1961) required the estimation of the free energy of hydrolysis of α-D-galactose 1-phosphate. This hexose phosphate only differs from α-D-glucose 1-phosphate in the configuration of the hydroxyl group at $C_{(4)}$, which is *para* and *trans* to the phosphate; the difference between the free energies of hydrolysis of these compounds is probably less than 0·1 kcal./mole (Angyal & McHugh, 1956). The free energy of hydrolysis of α-D-glucose 1-phos-

phate may be calculated from the equilibrium constants of the phosphoglucomutase reaction:

α-D-Glucose 1-phosphate
⇵
$(\alpha+\beta)$-D-glucose 6-phosphate

and of the reaction:

$(\alpha+\beta)$-D-Glucose 6-phosphate + water
⇵
D-glucose + orthophosphate,

catalysed by phosphatase.

Colowick & Sutherland (1942) measured the equilibrium constant of the phosphoglucomutase reaction at pH 7·5 and 30° in the presence of 8 mM-Mg^{2+}. This measurement has been repeated in the conditions used for the galactokinase reaction, namely at pH 7·0 and 25° in the presence of 25 mM-Mg^{2+}.

Meyerhof & Green (1949) and Guinodman (1954) have measured the equilibrium constant of glucose 6-phosphate hydrolysis, but values of the free energy of hydrolysis calculated from the two sets of results differ by about 0·7 kcal./mole. This equilibrium constant has been measured again at pH 7·0 and 25° with and without added Mg^{2+} ions.

A preliminary account of this work has been published (Atkinson *et al.* 1959).

MATERIALS

D-Glucose, sodium dihydrogen phosphate, disodium hydrogen phosphate and magnesium chloride were A.R. grade (British Drug Houses Ltd.).

Triphosphopyridine nucleotide (TPN), barium glucose 1-phosphate, barium glucose 6-phosphate, barium fructose 6-phosphate, glucose 6-phosphate dehydrogenase (Type II) and glucose oxidase were from the Sigma Chemical Co. Where necessary, barium salts were converted into sodium salts before use.

Alkaline phosphatase from calf intestinal mucosa and muscle enzyme paste for hexose phosphate determinations were described previously (Atkinson *et al.* 1961).

Phosphoglucomutase was purified from rabbit muscle (Najjar, 1955) up to the first crystallization; the material precipitating between 0·5 and 0·6 saturation of ammonium sulphate was used.

ANALYTICAL METHODS

Orthophosphate. This was determined colorimetrically after extraction as phosphomolybdate (Weil-Malherbe & Green, 1951).

Glucose. This was estimated manometrically with glucose oxidase (Keilin & Hartree, 1945) without addition of ethanol. Addition of catalase to the crude glucose-oxidase preparation did not change the observed oxygen uptake.

Glucose 6-phosphate. This was measured spectrophotometrically with TPN and glucose 6-phosphate dehydrogenase from yeast (Kornberg & Pricer, 1951). Whereas there was no reaction with glucose 1-phosphate, fructose 6-phosphate caused reduction of TPN owing to the presence of phosphohexose isomerase. The true value for glucose 6-phosphate was obtained by subtracting the fructose phosphate estimated colorimetrically with resorcinol (Ashwell, 1955). In the phosphoglucomutase equilibrium, the total fructose phosphate measured colorimetrically did not correspond to more than 3% of the total hexose 6-phosphate measured enzymically (see Table 1).

α-D-*Glucose 1-phosphate.* This was estimated by the addition of phosphoglucomutase to the glucose 6-phosphate dehydrogenase reaction mixture after oxidation of hexose 6-phosphates. It was also estimated as the difference between total hexose phosphates measured by Slater's (1953) procedure *B* (*a*) and hexose 6-phosphates measured with glucose 6-phosphate dehydrogenase. The results given in Tables 1 and 2 were obtained in the latter way. These results agreed closely with the increase in orthophosphate measured colorimetrically after hydrolysis of the equilibrium mixture in 0·1N-HCl at 100° for 10 min. ('acid-labile phosphate'; see Table 1).

EXPERIMENTAL AND RESULTS

Establishment of equilibria

With phosphoglucomutase. Solutions of magnesium chloride (500μmoles) in water (10 ml.) were mixed with glucose 6-phosphate (about 290μmoles), with glucose 1-phosphate (about 270μmoles) or with glucose 6-phosphate (about 21μmoles) and glucose 1-phosphate (about 21μmoles). The solutions were adjusted to pH 7·0 with either sodium hydroxide or hydrochloric acid, mixed with enough phosphoglucomutase to ensure equilibration within 24 hr., and finally adjusted to pH 7·0 and 20 ml. After incubation at 25° in the presence of toluene vapour for 72 hr., 2 ml. of 10N-hydrochloric acid was added. After 5 min. at 0°, the solution was brought to pH 7 with sodium hydroxide and then centrifuged to remove protein. The equilibrium concentrations of glucose 6-phosphate and of α-D-glucose 1-phosphate in the supernatants is shown in Table 1.

With phosphatase. Solutions of sodium phosphate (mono- and di-hydrogen forms) were mixed to give pH 7. Five reaction mixtures were prepared containing orthophosphate (about 3·3, 5·0, 4·9, 4·8 and 3·3 m-moles), glucose (3·0, 4·3, 4·8, 5·0 and 5·1 m-moles respectively) and magnesium chloride (0, 40, 100, 40 and 40μmoles respectively). The fourth and fifth also contained glucose 6-phosphate (20μmoles). After addition to each reaction mixture of enough phosphatase to catalyse hydrolysis of 0·4 m-mole of *p*-nitrophenyl phosphate/hr. at pH 9·5 and 20°, the solutions were adjusted to pH 7·0 and 20 ml. The solutions were kept at 25° under toluene vapour. After 12 days no further change in composition of the reaction mixtures could be detected. The phosphatase was shown to be still active after a further 4 days, at

Table 1. *Equilibrium constant of the phosphoglucomutase reaction at* pH 7·0 *and* 25° *with* 25 mM-Mg^{2+}

Methods are described in the text. Total hexose phosphates are the sum of glucose 6-phosphate, glucose 1-phosphate and fructose phosphate. $K = [(\alpha+\beta)$-D-glucose 6-phosphate$]/[\alpha$-D-glucose 1-phosphate], and $\Delta G' = -RT \ln K$. —, Represents below the limits of estimation.

	Equilibrium concentrations (mM)		
	From glucose 6-phosphate	From glucose 1-phosphate	From glucose 6-phosphate and glucose 1-phosphate
1. Total hexose phosphates	14·7±0·1 (5)	13·8±0·1 (6)	2·26±0·07 (5)
2. Glucose 6-phosphate plus fructose phosphate	13·9±0·4 (12)	12·9±0·2 (6)	2·15±0·03 (13)
3. Fructose phosphate	0·34	0·31	0·006
α-D-Glucose 1-phosphate [(1) − (2)]*	0·8	0·9	0·11
(α + β)-D-Glucose 6-phosphate [(2) − (3)]*	13·6	12·6	2·1
Acid-labile phosphate	0·6	0·9	—
K	17	14	19
$\Delta G'$ (kcal./mole)	−1·7	−1·6	−1·7

* These values are used in the calculation of K; see Analytical Methods section.

Table 2. *Equilibrium constant of glucose 6-phosphate hydrolysis at* pH 7·0 *and* 25°

Methods are described in the text. $K = [(\alpha+\beta)$-D-glucose$]$ [orthophosphate$]/[(\alpha+\beta)$-D-glucose 6-phosphate].

	Equilibrium concentrations (mM)				
Mg^{2+} (initial concn.)	0	2	5	2	2
Orthophosphate	163±6 (3)	248	246	240±1 (3)	167±2 (3)
(α + β)-D-Glucose	151±6 (3)	214	239	248	253
(α + β)-D-Glucose 6-phosphate	0·076	0·201	0·224	0·319±0·009 (4)*	0·152*
10^{-2} K	3·2	2·6	2·6	1·9	2·8
$\Delta G'$ (kcal./mole)	−3·4	−3·3	−3·3	−3·1	−3·3

* Initial concn. was 1 mM.

which time the solutions were finally adjusted to pH 7·00 ± 0·02. One day later the reaction was stopped with hydrochloric acid, and samples were obtained for analysis as described above. The equilibrium concentrations of glucose, orthophosphate and glucose 6-phosphate are given in Table 2.

DISCUSSION

The equilibrium constant of the reaction catalysed by phosphoglucomutase is 17 ± 2 at pH 7·0 and 25° in the presence of 25 mM-Mg^{2+} (Table 1). Since glucose 1-phosphate and glucose 6-phosphate have similar affinities for H^+ ions (Oesper, 1951) and probably also for Mg^{2+} ions, the equilibrium constant of their interconversion should vary little with changes of pH and concentration of Mg^{2+} ions. The value found here is identical with that found at pH 7·5 and 30° with 8 mM-Mg^{2+} (K, 17) by Colowick & Sutherland (1942). The apparent free-energy change of this reaction is therefore −1·7 ± 0·1 kcal./mole.

The equilibrium constant of hydrolysis of glucose 6-phosphate at pH 7·0 and 25° shows little change on increasing the concentration of Mg^{2+} ions from 0 to 5 mM; the corresponding values of apparent free energies of hydrolysis differ by less than 0·1 kcal./mole (Table 2). The mean equilibrium constant for five experiments is 260 ± 50 and thus the apparent free energy of hydrolysis of (α + β)-D-glucose 6-phosphate is −3·3 ± 0·1 kcal./mole. From equilibrium concentrations in this reaction reported by Meyerhof & Green (1949), Burton & Krebs (1953) calculated a value of −3·1 kcal./mole for the free energy of hydrolysis of glucose 6-phosphate at pH 7 and 25°. The total concentrations of reactants in the equilibria studied by Meyerhof & Green (1949), which were established at pH 8·5 and 5·8, were about ten times those shown in Table 2. In view of these differences in experimental conditions, the calculated free energies of hydrolysis from these two studies are in good agreement. Guinodman (1954) studied the same equilibrium and from his results calculated a value of −2·6 kcal./mole at pH 7 and 25°. The studies reported here, in which specific enzymic methods of estimation have been used, do not support this value.

Summation of the apparent free-energy changes of the phosphoglucomutase and phosphatase reactions (Tables 1 and 2) give the apparent free energy of hydrolysis of α-D-glucose 1-phosphate as −5·0 kcal./mole at pH 7·0 and 25° in the presence of Mg^{2+} ions.

None of the equilibrium concentrations discussed here have been corrected for variation of the activity coefficients from unity, but the absence of any apparent dependence of the constants on reactant concentrations suggests that the apparent free energies of hydrolysis calculated here differ little from the true free energies of hydrolysis.

SUMMARY

1. Specific enzymic methods were used to estimate the equilibrium concentrations of $(\alpha+\beta)$-D-glucose 6-phosphate and of α-D-glucose 1-phosphate in the products of the reaction catalysed by muscle phosphoglucomutase at pH 7·0 and 25° with 25 mM-Mg^{2+}. The apparent equilibrium constant, $[(\alpha+\beta)$-D-glucose 6-phosphate$]/[\alpha$-D-glucose 1-phosphate], was 17 ± 2 and the apparent free energy of phosphoryl transfer ($\Delta G'$) was $-1{\cdot}7 \pm 0{\cdot}1$ kcal./mole.

2. Specific enzymic methods were also used to estimate the equilibrium concentrations of $(\alpha+\beta)$-D-glucose and of $(\alpha+\beta)$-D-glucose 6-phosphate in the products of hydrolysis of glucose 6-phosphate catalysed by intestinal alkaline phosphatase. Orthophosphate was estimated as phosphomolybdate. At pH 7·0 with 5 mM-Mg^{2+} the apparent equilibrium constant $[(\alpha+\beta)$-D-glucose$]$ [orthophosphate$]/[(\alpha+\beta)$-D-glucose 6-phosphate] was 260 ± 50, and the apparent free energy of hydrolysis was $-3{\cdot}3 \pm 0{\cdot}1$ kcal./mole. There was little change in the apparent equilibrium constant with increasing concentration of Mg^{2+} ions between 0 and 5 mM at pH 7·0.

3. From these results, the apparent free energy of hydrolysis of α-D-glucose 1-phosphate is $-5{\cdot}0$ kcal./mole at pH 7·0 in the presence of Mg^{2+} ions.

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The Chemistry of Connective Tissues

6. THE CONSTITUTION OF THE CHONDROITIN SULPHATE–PROTEIN COMPLEX IN CARTILAGE†

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It has been known for some time that a large part of the chondroitin sulphate of cartilage is combined with a protein which is not derived from collagen (Shatton & Schubert, 1954; Partridge & Davis, 1958*a*; Muir, 1958). It appears to be this entity which enters into loose combination with collagen fibres and soluble collagen to give the tissue its characteristic physical properties and insolubility. Several different procedures have been used for the extraction from cartilage of material containing chondroitin sulphate and protein. The most effective of these have included the use of 10% calcium chloride solution (Meyer & Smyth, 1937; Mathews & Dorfman, 1953; Mathews, 1956;

† Part 5: Thomas & Partridge (1960).

Muir, 1958), extraction with warm aqueous 50% phenol (Westphal, 1952; Partridge & Davis, 1958*b*), the use of a preliminary heat treatment to effect the release of the complex by thermal shrinkage of the collagen fibres (Partridge, 1948) or extraction with 30% potassium chloride solution made mildly alkaline by the addition of potassium carbonate (Einbinder & Schubert, 1950; Partridge & Davis, 1958*a*).

Since the physical properties of the products differ widely according to the method of extraction it is probable that these extraction conditions, and the subsequent purification procedures which are necessary to remove collagen, together result in some degree of denaturation or chain rupture. However, quite recently Malawista & Schubert (1958) found that if either fresh or dried cartilage powder is homogenized vigorously with a very large volume of water at temperatures below 15°, a large part of the material passes into solution. The solution is very viscous and about half the dry matter consists of collagen in a soluble and apparently undenatured form, which is readily precipitated by the addition of 2 vol. of ethanol. The chondroitin sulphate–protein complex remains in solution but is precipitated on addition of potassium acetate to the ethanolic solution.

In the present work two sources of the chondroitin sulphate–protein complex were used: material extracted from dried powdered cartilage by homogenizing in a large volume of water and material extracted in the same way from fresh cartilage slices. The complex prepared from dried cartilage proved to be slightly degraded, but because of the very mild conditions used it is thought that the product from fresh cartilage was substantially in the native state.

Evidence has already been given (Partridge & Davis, 1958*a*; Muir, 1958) that the protein component of the complex is firmly bound, and probably chemically combined with the polysaccharide residue, and other evidence is given in the paper which follows (Partridge & Elsden, 1961). In order to obtain further information about the structure of the complex it was decided to compare the polysaccharide chain weight (as measured by estimating the reducing power due to the terminal residue) with molecular weights determined osmotically, and to study both the native complex and products formed from it by controlled degradation with enzymes.

METHODS

Preparation of cartilage powder. Fresh cartilage from nasal septa of cattle was freed from adhering connective tissue and finely chopped with a scalpel. The pieces were washed twice with 1% (w/v) NaCl solution by gently shaking and were then rinsed in water and put into acetone. After standing for some time in two or three changes of solvent they were dried in air at 70° and ground in a hammer mill through a sieve with circular apertures 0·56 mm. in diam.

Preparation of chondroitin sulphate–protein complex from fresh cartilage slices. The extraction procedure was based on the method described by Malawista & Schubert (1958). Fresh cartilage was taken as soon as possible after slaughter from cattle 2–3 years old. After removal of adhering connective tissue the cartilage was cut into strips, rapidly washed with 1% (w/v) NaCl and stored at −20°. Batches (10 g.) were taken from storage and cut into slices about 2 mm. thick, the slices being dropped at once into 300 ml. of ice-cold water. The suspension was then vigorously homogenized in an Ato-Mix homogenizer (Measuring and Scientific Equipment Ltd., London) at 12 000 rev./min. for 5 min. or until the temperature rose to 15°. The glass container with the suspension was then cooled in an ice bath to 2° and the homogenization was repeated for a further 5 min. This was repeated twice more, the total time of homogenization being 20 min. at 2–15°. The suspension was centrifuged and 2 vol. of ethanol was added to the supernatant. The mixture was allowed to stand for 2 hr., when Celite 545 (Johns–Manville, London) (10 g.) was stirred in and the suspension centrifuged for 20 min. at 2500 rev./min. The supernatant was filtered, first through a glass-wool plug and then through a fluted filter paper (Whatman no. 54). Potassium acetate (10 g.) was then added to the solution and the precipitate collected by centrifuging. It was washed three times with 75% (v/v) ethanol to remove potassium acetate and then taken up in 40 ml. of water. The solution was colourless, clear and very viscous. It was dialysed in cellophan overnight at 2° against a large volume of distilled water and stored in the frozen state at −20°.

Preparation of chondroitin sulphate–protein complex from dried cartilage powder. The powder (2·5 g.), which had been stored at room temperature for several weeks, was rehydrated by allowing it to stand with 300 ml. of water for 1 hr. The suspension was cooled and extracted with the Ato-Mix homogenizer in the same way as with fresh cartilage slices. As judged from the hydroxyproline content, the chondroitin sulphate–protein complex isolated by precipitation with potassium acetate contained 10–15% of collagen in different preparations and further purification was effected by treatment with a carboxylic acid ion-exchange resin (Partridge & Davis, 1958*a*). The resin (Amberlite CG 50, 100–200 mesh) was prepared by treating it with 2N-NaOH and washing by decantation. This was followed by treatment with 2N-HCl and further washing. It was then equilibrated with 0·1M-potassium acetate of pH 5·0 by stirring with several successive volumes of buffer. The treated resin (dry wt. 1 g.) was added to 180 ml. of an approx. 1% (w/v) solution of the chondroitin sulphate–protein complex and 20 ml. of M-potassium acetate buffer (pH 5) was added. The mixture was gently agitated in a mechanical shaker for 1 hr. and the resin was then separated by centrifuging. The process was repeated once more with a fresh batch (1 g.) of resin. After dialysis to remove buffer the complex recovered by drying in the frozen state had a hydroxyproline (colorimetric) content of 0·5%, showing that contamination with collagen was not more than about 3·5%.

Treatment of the chondroitin sulphate–protein complex with papain. Solutions of the chondroitin sulphate–protein complex were found to be too viscous for accurate pipetting and were therefore weighed out. A portion (30 g.) of an approx. 1% (w/v) solution, which had been thawed at room temperature after storage at −20°, was mixed with 24 ml. of water and 6 ml. of 0·2M-potassium phosphate buffer, pH 6·5. Cysteine hydrochloride (1·2 m-moles) and two or three crystals of benzoic acid were then dissolved in the solution. Crystalline papain (3 mg.; Kimmel & Smith, 1954) was added and the pH readjusted to 6·5 by the addition of N-NaOH. The solution was incubated for 24 hr at 37° and dialysed for 24 hr. against repeated changes of distilled water. A light flocculent precipitate formed during dialysis and was removed by filtration. A portion (40 ml.) of the clear colourless solution was stored at −20° for osmotic-pressure experiments, and the remainder (46 ml.) was reserved for dry-weight determination (by freeze-drying) and for estimation of reducing power.

Treatment of the chondroitin sulphate–protein complex with hyaluronidase. The action of hyaluronidase after varying periods of incubation from 6 hr. to 3 days was investigated as follows. The solution of chondroitin sulphate–protein complex which had been stored at −20° was thawed, thoroughly mixed and its concentration was determined by freeze-drying a sample. The viscous solution contained about 1% of solids and 24 ml. was measured out by weighing. This was mixed with 6 ml. of buffer solution (0·5M-potassium acetate, pH 4·42, containing 0·75M-NaCl). Part of this solution (10 ml.) was used as an untreated control and the remainder (20 ml.) was incubated with ovine hyaluronidase (Benger Laboratories Ltd., 4 mg.) at 37° under toluene. Control solutions containing only hyaluronidase and buffer were also set up. In the experiments in which the incubation time was longer than 24 hr. a further quantity of hyaluronidase (2 mg.) was added after each 24 hr. period.

The control solutions were dialysed against distilled water and the volumes were measured. Part of the untreated control solution was then used for determination of reducing power (glucose equivalent) and the remainder dried from the frozen state for determination of dry matter, ash and nitrogen. The reducing power of the solution after treatment with hyaluronidase was estimated and the glucose equivalent of the incubated enzyme plus buffer control solution was subtracted. The remainder of the solution was adjusted to pH 8 and ultrafiltered through the highly permeable membrane described. Ultrafiltration was allowed to proceed until the volume in the sac was 10–15% of the original volume. Water was then added to make up the original volume and the solution refiltered. This was repeated four times. To test the efficiency of the ultrafiltration process the distribution of reducing power in the successive ultrafiltrates was determined. In one experiment (incubation time 24 hr.) the total glucose equivalent of the solution after treating with hyaluronidase was 7·50 mg. The first, second, third and fourth ultrafiltrates contained respectively 4·74, 1·16, 0·30 and 0·12 mg. of glucose equivalent (total 6·32 mg.), and the glucose equivalent remaining in the sac was 1·44 mg. The small quantity of diffusible reducing substance remaining in the sac was removed by dialysis against a large volume of water. The ultrafiltrates were combined and the volume was measured; the nitrogen content and glucose equivalent were determined on the solution. The solution was then dialysed through cellophan and a sample from the cellophan sac was dried from the frozen state for determination of nitrogen and ash. The polysaccharide content of the total ultrafiltrate was then calculated from the nitrogen content of the solution and its volume.

The solution remaining in the ultrafiltration sac was free from buffer salts. The reducing power was determined on the solution and a portion was dried from the frozen state for determination of dry weight, nitrogen and ash. The remainder was used for determination of molecular weight by measurement of osmotic pressure.

Purified potassium chondroitin sulphate. The sample was prepared by alkaline degradation and purified by the use of ion-exchange resin as described by Partridge & Davis (1958*a*) [Found: N, 2·83; hexosamine (colorimetric), 24·7; uronic acid as galacturonic acid (carbazole), 27·1; ash (as K_2SO_4), 30·2%].

Treatment of purified potassium chondroitin sulphate with hyaluronidase. Potassium chondroitin sulphate (2 g.) prepared as described above was dissolved in 200 ml. of 0·1M-potassium acetate containing 0·15M-NaCl and the pH was adjusted to 4·55 by the addition of acetic acid. Ovine hyaluronidase (40 mg.) was then added and the mixture incubated for 120 hr. at 37° under toluene. The solution was then dialysed for 72 hr. in Visking tubes against repeated changes of distilled water. Part of the solution of non-diffusible substances was dried from the frozen state for dry-weight determination and analysis. The total yield was 1·28 g. (anhydrous) (Found: N, 3·24; glucose equivalent, 8·36%). The increased nitrogen content compared with the starting material (N, 2·83%) could be accounted for by the enzyme added.

Estimation of hexosamines. Total hexosamine was estimated colorimetrically by the method of Elson & Morgan (1933) as modified by Rondle & Morgan (1955) after hydrolysis with 4N-HCl for 16 hr. in a sealed tube at 100°. Glucosamine and galactosamine were estimated separately by the chromatographic method of Gardell (1953) after hydrolysis under the same conditions as were used for the colorimetric method. In some experiments glucosamine and galactosamine were determined by the use of a 9 mm. × 15 cm. Moore, Spackman & Stein (1958) column and by elution with 0·35M-sodium citrate buffer, pH 5·26, at 50° (cf. Eastoe, 1954). The peaks due to the hexosamines were estimated by the use of the ninhydrin reagent of Yemm & Cocking (1955).

Estimation of hydroxyproline. As a rapid method for use where the content of collagen was high, the colorimetric method of Newman & Logan (1950) as modified by Miyada & Tappel (1956) was employed. This method gave high results when used to estimate the hydroxyproline content of samples containing less than about 5% of collagen. This was probably due to interference from tyrosine or tryptophan in the non-collagenous protein (see Miyada & Tappel, 1956). For final analysis of samples with a low hydroxyproline content the material was refluxed with 6N-HCl for 24 hr. and the hydroxyproline peak separated chromatographically with a 15 cm. Moore & Stein (1951) column. The column was equilibrated and eluted at 37° with 0·1M-sodium citrate buffer, pH 3·0, and concentrations in the peaks were measured by use of the ninhydrin reagent of Yemm & Cocking (1955).

Estimation of reducing power due to the terminal residue in chondroitin sulphate chains. The measurement of reducing power by standard methods proved to be subject to a number of errors when applied to chondroitin sulphate. As a model substance the oxidation of the purified preparation of potassium chondroitin sulphate described above was studied, and three methods of determining the reducing power were investigated: (1) The Somogyi (1937) copper-reduction method as modified by Nelson (1944). (2) The Hagedorn & Jensen (1923) alkaline-ferricyanide method as modified by Hanes (1929). (3) The alkaline-iodine method as described by Weissmann, Meyer, Sampson & Linker (1954).

Results from the iodine method were consistently about 50% higher than those from copper reduction, but since Weissmann *et al.* (1954) have shown that *N*-acetylhyalobiuronic acid required 3·2 equiv. of I_2/mol. instead of 2 equiv./mol. this result is understandable. The alkaline-ferricyanide method gave results which were high and erratic and accordingly all further determinations were carried out by copper reduction. The essential validity of the method as applied to pure preparations of chondroitin sulphate was shown by analysis of a sample which had been dialysed exhaustively in cellophan. The chain weight by copper reduction was 16 600, and the molecular weight, determined osmotically, was 16 100. The procedure finally adopted was as follows.

Samples of the solution to be estimated, containing up to 0·3 mg. of glucose equivalent, were placed in 17 mm. test tubes provided with calibration marks at 2 and 25 ml. If the solution was very viscous, as with the native chondroitin sulphate–protein complex, the solution was weighed in the tubes. The volume was then made up to the 2 ml. mark with water. Usually three pairs of duplicates, containing solutions of different concentration, were taken for each estimation. The Somogyi copper reagent A (2 ml.; Nelson, 1944) was then added and the tubes were heated in a boiling-water bath for 10 min. The tubes were not disturbed whilst in the bath. After cooling, 2 ml. of the Nelson (1944) arsenomolybdate reagent was added and the tubes were well shaken. They were then made up to the 25 ml. mark and read with the Hilger Spekker absorptiometer with 1 cm. cells and an Ilford no. 604 green filter. A calibration curve was constructed for glucose with quantities in the range 0·03–0·3 mg. and the reducing power of the test solution was expressed in terms of mg. of glucose equivalent/ml. When solutions which contained buffer or added enzyme were examined, the glucose equivalent of the buffer or the enzyme was determined separately and subtracted.

Preparation of membranes. The membrane of high permeability used for ultrafiltration was made from a solution composed of 4 g. of pyroxylin (Imperial Chemical Industries Ltd.; reference no. HL 120/170), 50 ml. of absolute ethanol, 50 ml. of anhydrous ether and 18 ml. of ethylene glycol. Five coats of this solution were poured over a glass tube rotated at 15 rev./min. The tube was dried for 20 hr. at 18° in a room with a humidity of 58%. The membrane was 13·5 cm. long, 2·22 cm. in diam. and 0·032 cm. thick. At a pressure of 340 mm. Hg the rate of ultrafiltration of water was 0·77 ml./min. at 16°. This rate is comparable with the rate calculated for a membrane with cylindrical pores of diameter 212 Å. The membranes for the osmotic experiments were prepared as described by Adair (1949). All calculations of ultrafiltration rates given later, represented by the symbol q', refer to a temperature of 16°, a pressure of 441 mm. Hg and an area of 28·4 cm.^{2}.

RESULTS

Properties of the complex extracted from dried cartilage powder and from fresh cartilage

In fourteen experiments the yield of crude chondroitin sulphate–protein complex obtained from 2·5 g. of dried cartilage powder varied from 0·39 to 0·56 g. The hydroxyproline content of the product varied from 1·46 to 2·34%, indicating contamination with collagen to the extent of 10–15%. After a single treatment with the resin IRC-50 the hydroxyproline content (colorimetric) was decreased to 0·5–0·9%, but further resin treatment was ineffective in decreasing the hydroxyproline content of any sample below 0·3–0·5%. This corresponded to a maximum of 3·5% of collagen. [Analysis of a bulked sample of the product from 10 preparations after vacuum–ice drying showed: N, 4·83; hydroxyproline (colorimetric), 0·29; glucosamine, 3·3; galactosamine, 19·4; ash (as K_2SO_4), 25·5; uronic acid (carbazole), 22·4%.]

The course of extraction from fresh cartilage was rather different. The yield of complex from eighteen batches, each of 10 g. of cartilage slices (about 2·5 g. dry wt.), varied from 0·326 to 0·420 g. Separation of soluble collagen by precipitation with ethanol was more nearly quantitative than with the extracts of dried cartilage, and the hydroxyproline content (colorimetric) of the samples was in the range 0·31–0·55%. Treatment with ion-exchange resin (IRC-50) did not decrease this figure and no further purification was attempted. A representative sample of the chondroitin sulphate–protein complex, drawn from twelve batches, had: N, 5·72; glucosamine, 3·4; galactosamine, 20·2; ash (as K_2SO_4), 22·68; uronic acid (carbazole), 20·0%.

It is known that, in addition to chondroitin sulphate A, bovine hyaline cartilage contains smaller quantities of other polysaccharides: in particular chondroitin sulphate C and keratosulphate (Meyer, Linker, Davidson & Weissmann, 1953). It is commonly accepted that these two polysaccharides are also combined in some way with protein but it is not known if they exist as independent polysaccharide–protein complexes or if two or more polysaccharide chains of different types may be attached to the same protein. At present no means of separating the macromolecular complexes are known to the authors and, in view of the difficulty of avoiding partial degradation even with very mild treatments, no extensive fractionation of the extracted complex was

Table 1. *Number-average chain weight (Cn) and number-average molecular weight (Mn) of preparations from dried cartilage powder*

	N (%)	*Cn*	*Mn*
(A) Water extract	5·13	18 000	140 000 ± 20 000
(B) (A) treated with papain	2·86	18 100	14 000 ± 3 000
(C) Purified chondroitin sulphate (K^+ salt) prepared by alkaline degradation	2·83	16 600	16 100 ± 1 000
(D) (C) treated with hyaluronidase followed by dialysis			
Preparation 1	3·24	2 150	3 800
Preparation 2	3·28	2 860	4 900

Table 2. *Chain weight (Cn), molecular weight (Mn) and number of reducing residues per molecule (R) of preparations from fresh cartilage*

Values (*) are calculated for the potassium salt.

Preparation	Source	N (%)	*Cn*	*Mn*	*R*
E1	Water extract	5·25	43 000	$1–5 \times 10^6$	23–115
E2	Water extract	5·72	53 000	$1–5 \times 10^6$	19–95
F1	Preparation E1 treated with papain	2·87	51 000	28 500 ± 2 000*	0·56
F2	Preparation E1 treated with papain	2·87	50 000	27 500 ± 1 000*	0·55
G	Preparation E2 treated with hyaluronidase followed by ultrafiltration	9·85	7 730	170 000 ± 2 000*	23 ± 2

attempted. It has not been considered necessary to distinguish between chondroitin sulphate A and chondroitin sulphate C in this work since the two polysaccharides are closely related isomers. In the paper that follows (Partridge & Elsden, 1961) some evidence is presented in favour of the suggestion that keratosulphate forms an integral part of the complex containing chondroitin sulphate. The content of glucosamine in the ethanol-fractionated water extract is less than 15% of the total hexosamine, showing that the keratosulphate content of the total polysaccharide could not be greater than about 15%.

Determination of chain weight (Cn) by measurement of reducing power due to the terminal residue

In applying the method of chain-weight determination described above to the degradation products of the chondroitin sulphate–protein complex, there were several sources of error which appeared to have little importance with the pure polysaccharide:

(1) Losses in reducing power due to Maillard (1912) reactions. This error was reduced by avoiding the storage of dried products. Samples for analysis were stored as solutions in the frozen state at $-20°$.

(2) Loss of reducing power due to the use of ethylenediaminetetra-acetate (EDTA) in the experiments with papain. Ogston & Sherman (1959) have shown that EDTA in the presence of trace amounts of heavy metals probably acts as an oxidation catalyst. The use of EDTA proved to be unnecessary and was avoided in further experiments.

(3) Loss of reducing power due to the use of ether or other peroxide-containing solvents.

(4) Errors due to the reducing power of the protein moiety, which appeared to be small.

The procedures for the preparation of samples for analysis described under Methods were designed to avoid these sources of error so far as was possible. Replicate determinations of reducing power on the same sample agreed to within ±2% but determinations on different samples of chondroitin sulphate–protein complex showed ±6% variation in one series.

The number-average chain weight (*Cn*) of a preparation was calculated from the expression

$$Cn = \frac{180 \times 1000}{\text{glucose equivalent}},$$

where 180 is taken as the molecular weight of glucose and 'glucose equivalent' is defined as the reducing power of 1 g. of sample expressed as mg. of glucose. The results obtained with extracts of dried cartilage powder are summarized in Table 1 and those with extracts from fresh cartilage in Table 2. The values for *Cn* in the Tables are not corrected for the varying amounts of protein in the different preparations.

Determination of molecular weight (Mn) from osmotic-pressure experiments

The majority of the measurements of osmotic pressure were made with a high concentration of diffusible electrolyte and very low concentrations, usually less than 0·2 g. of chondroitin sulphate/100 ml., in order to decrease the partial pressure,

π_i, due to the net excess of diffusible ions inside the membrane. Formula (1), used for calculating π_i for ideal solutions, was derived from more general formulae published by Adair & Robinson (1930):

$$\pi_i = RT\,(C_e\alpha)^2/4I. \qquad (1)$$

(R, Gas constant; T, absolute temperature.) Since all pressures are given in mm. Hg at 0°, the product RT is equal to 17 034. The symbol C_e denotes the equivalent concentration of the potassium or sodium salt of chondroitin sulphate, discussed below, and α denotes the degree of ionization of this salt. The symbol I denotes the ionic strength of the diffusible electrolytes in the external solution or dialysate. Most of the colloidal solutions were dialysed with large volumes of an external solution made up with 0·015 M-Na_2HPO_4, 0·005 M-NaH_2PO_4 and 0·5 M-NaCl. This external solution has pH 6·96 at 1° and I 0·55.

Determination of α. It is difficult to make direct measurements of α for solutions with high concentrations of diffusible salts, and therefore measurements were made with solutions in water. One solution, with a final concentration of 0·889 g. of the potassium salt of chondroitin sulphate/100 ml., gave an osmotic pressure of 288 mm. Hg at 0° and an 'apparent molecular weight' of 526. A more dilute solution, with 0·49 g./100 ml., gave a pressure of 164·1 mm. and an apparent molecular weight of 509. The term apparent molecular weight denotes the ratio M/ϕ, where M is the average molecular weight and ϕ is a type of osmotic coefficient, which represents the ion-pressure difference π_i and deviations from the ideal solution laws. The ratio M/ϕ is calculated from formula (2):

$$M/\phi = 10RT(C/\pi) = 170\,342\,(C/\pi). \qquad (2)$$

(π, Osmotic pressure, mm. Hg at 0°; C, g. of chondroitin sulphate/100 ml. of solution.) Values of M/ϕ for the solutions of the potassium salts of chondroitin sulphate in water, 526 and 509, are small compared with the mean molecular weight (M 14 300) estimated for solutions with I 0·55; thus the factor ϕ is large and the greater part of the observed osmotic pressures of the solutions in water is due to K^+ ions. It may be noted that the observed values of M/ϕ are approximately twice the equivalent weight, 268, calculated on the assumptions that the carboxyl groups and the sulphate groups are fully ionized. If the degree of ionization (α) be 0·5, and the equivalent weight be 268, the product $C_e\alpha$ in equiv./l. is 0·0187C.

If it is assumed that $C_e\alpha$ is 0·0187C when I is 0·55 it follows that $\pi_i = 2{\cdot}6C^2$. For a dilute solution the pressure π_i is very small (0·026 mm. Hg when C is 0·1 g./100 ml.) and for the purpose of calculating molecular weight by the modified form of van't Hoff's law (equation 3) measurements were made on solutions where C was usually less than 0·2:

$$\pi = \pi_p + \pi_i = (10RT/M)\,C + KC^2. \qquad (3)$$

(π_p, Pressure due to colloidal ions; KC^2, a small term, proportional to C^2, which represents the term π_i and the effects of deviations from the ideal solution laws.) The value 2·6 is not exact and the range of application of equation 3 should be limited to solutions where C is less than 0·2 and M is less than 100 000 and π_p is much greater than π_i. Preliminary experiments on solutions with higher concentrations (0·3–0·8 g./100 ml.) gave variations in M greater than the experimental errors.

With the samples of chondroitin sulphate prepared by dissociation with alkalis or by papain treatment the values of π_i were much smaller than the observed osmotic pressures, when I was 0·55, and it was thus possible to estimate their molecular weights within the limits given (Tables 1 and 2). In other preparations the molecular weights were very high or very low. Details of the treatment of these samples are given below.

Complex prepared from fresh cartilage. From viscosity and light-scattering data, Mathews & Lozaityte (1958) report, for the chondroitin sulphate–protein complex, weight-average molecular weights of 4×10^6 or more. A solution of the complex prepared from fresh cartilage with a final concentration of 0·17 g./100 ml. gave an osmotic pressure of 1·03 mm. of toluene at 0·68°, equivalent to 0·067 mm. Hg at 0°. The apparent molecular weight M/ϕ is 434 000. The observed osmotic pressure, 0·067 mm., is only slightly greater than the partial pressure, π_i 0·051 mm., estimated by assuming that the equivalent weight is 268, the equivalent concentration is 0·0052 and α is 0·5. The result is consistent with that of Mathews & Lozaityte since M/ϕ is much less than M when the greater part of the observed osmotic pressure is due to inorganic ions.

Complex prepared from dried cartilage. A sample of the complex prepared from dried cartilage gave a pressure of 4·0 mm. toluene at 0·7°, equivalent to 0·26 mm. Hg at 0°, for a solution containing 0·155 g./100 ml. This pressure is larger than the pressure of 0·06 mm. estimated for the diffusible ions in a solution containing the same concentration of chondroitin sulphate. The ratio M/ϕ is 102 000, and the mean molecular weight M is approx. 140 000 ± 20 000.

Purified sodium chondroitin sulphate prepared by alkaline degradation. Samples of chondroitin sulphate, degraded by alkalis, gave much higher pressures and smaller molecular weights. One solution (0·1 g./100 ml.) gave a pressure of 16·7 mm.

toluene at 0·7°, or 1·08 mm. Hg at 0°. The ratio M/ϕ was 15 700 and the mean molecular weight M was 16 100. A solution with 0·369 g./100 ml. gave a pressure of 4·31 mm. Hg and a molecular weight of 15 900. A membrane of permeability q'' 0·0116 was used for these measurements.

Another sample prepared by dialysis with a more permeable membrane (q'' 0·0805) gave an average molecular weight of 19 000. Comparisons of initial and final concentrations showed that about 30% of the chondroitin sulphate had diffused across the membrane or had been adsorbed by the membrane in the course of 7 days. These losses of chondroitin sulphate were observed with solutions of high ionic strength (I 0·55). A membrane of high permeability (q'' 0·164) retained chondroitin sulphate for months, when water was used as solvent.

Purified chondroitin sulphate treated with hyaluronidase. Samples of chondroitin sulphate which had been degraded by alkalis and then digested with hyaluronidase contained substances which diffused across most collodion membranes. The apparent osmotic pressure π^* given by these membranes is the pressure at which gains in volume due to osmosis are balanced by losses due to ultrafiltration of solvent and leakage of the solute. This pressure π^* may be less than the pressure π given by an ideal membrane impermeable by the solute. Losses of solute and apparent osmotic pressures were influenced by the properties of the membranes.

An increase in the permeability for water, symbolized q'', was correlated with a reduction in the pressure π^* and an increase in the uncorrected molecular weight M^* calculated from π^*. The smallest value of M^*, 3052, was obtained with the least-permeable membrane with an ultrafiltration rate of 0·0041 ml./min. A value of 3587 was obtained with the second membrane, with an ultrafiltration rate of 0·011 ml./min. The maximum value for M^*, 4875, was given by the third membrane with an ultrafiltration rate of 0·0258 ml./min. These figures are useful as an upper limit. The corrected values must be smaller than M^*.

Comparison of chain weight and osmotic molecular weight

Preparations from dried cartilage. The results of the osmotic-pressure experiments carried out with preparations from dried cartilage powder are compared with the determinations of chain weight in Table 1. The initial chondroitin sulphate–protein complex had *Mn* about 140 000 and the polysaccharide chain weight was only 18 000. After papain treatment there was a decrease in nitrogen content due to removal of the protein moiety; the polysaccharide chain weight remained about the same but the osmotic molecular weight was decreased to a figure closely approximating to the chain weight. This suggests that the chondroitin sulphate–protein complex is composed of a series of chains of chondroitin sulphate, linked together by the protein to form a larger molecule. The chain weight of the purified chondroitin sulphate agreed well with its molecular weight and the two values lie fairly close to those for the complex after papain treatment. This might suggest that there is little chain rupture during the purification of chondroitin sulphate by treatment with alkali, but in fact the preparation was extensively dialysed through Visking tubing and considerable losses of material were observed owing to diffusion of chain fragments through the membrane.

When the purified chondroitin sulphate was treated with hyaluronidase and dialysed through cellophan about 36% of its weight was lost by diffusion through the membrane. The remainder (preparation 1) consisted of material of *Cn* 2150. The osmotic-pressure determinations on this material gave a rather higher figure for *Mn* and since it was observed that there were losses of chondroitin sulphate through the osmotic membrane it was thought that the higher value was due to loss of material of lower molecular weight during osmotic equilibration. Accordingly the experiment was repeated with a new preparation (preparation 2 in Table 1), but this time *Cn* was determined on the material remaining in the sac after conclusion of the osmotic experiment. *Cn* so determined was rather higher, 2860, but the agreement with *Mn* on the same preparation was still not as close as would be expected by taking into account the known errors.

Preparations from fresh cartilage slices. The values obtained for the osmotic molecular weight of samples of chondroitin sulphate–protein complex from dried cartilage powder were considerably lower than several estimates given in the literature for the weight- and number-average molecular weights of material extracted from fresh cartilage. The possibility that the process of drying, milling or storing powdered cartilage had resulted in some degradation was therefore considered. Water extracts were made by the use of the homogenizer from sliced fresh cartilage obtained from selected young animals. The extracts were found to be more viscous than those from dried material, and it was also found that, provided that the extraction was carried out below 15°, the collagen separated very sharply on the addition of ethanol.

A similar set of values for *Cn* and *Mn*, obtained with material from fresh cartilage, is given in Table 2. The osmotic molecular weight for these preparations ($1–5 \times 10^6$) was much higher than for those obtained by extraction of dried cartilage. The values for *Cn* in Table 2 were determined on the solution remaining in the sac after the osmotic

experiments, in order to avoid the possibility of error due to the loss of low-molecular-weight material by adsorption on the membrane or by diffusion through it. After treatment with crystalline papain *Mn* fell to 28 000 but *Cn* remained substantially the same at 50 000. Since the molecular weight cannot be less than the chain weight, and both are number averages, it was thought that the value for the apparent chain weight (*Cn*) may be high due to the presence of chains in which the terminal reducing residue was masked.

The masking of terminal groups by oxidation during the course of treatment with papain readily occurs if EDTA is present and may be catalysed by traces of heavy metals. Thus when the reaction was carried out in the presence of cysteine but not EDTA the results of a number of determinations of *Cn* on different preparations were in the range $45–55 \times 10^3$, but with EDTA also present the results were erratic and values up to 85×10^3 were found. It is thought possible that cysteine also promotes heavy-metal catalysis to a lesser extent and that the true value of *Cn* may be rather lower than 50 000.

Experiments with hyaluronidase

After treatment of the native complex with hyaluronidase, followed by ultrafiltration, the chondroitin sulphate content of the complex fell to approx. 30 %. The chain weight (uncorrected for protein content) fell to 7730 but the osmotic molecular weight (170 000) remained at more than 20 times this value. In the last column of Table 2, calculated values for the number of reducing residues (*R*) in the molecule for each preparation are given. For the native complex the range for *R* is 19–115; for the papain-treated preparations the values for *R* are less than unity owing to the apparent masking of the reducing terminals of some of the chains; for the hyaluronidase-treated preparation, which contains a very large proportion of protein, *R* is 23 ± 2. It thus appears that the effect of hyaluronidase is to decrease the length of the chondroitin sulphate chains to points near the protein core, forming a new unit of *Mn* 170 000 which bears 23 short polysaccharide chains.

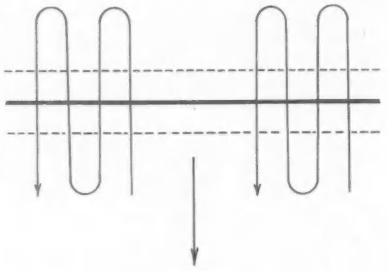

Fig. 1. Action of hyaluronidase on a model molecule in which the chondroitin sulphate chains are combined at several points to a protein core. The protein core is depicted by a heavy line and the arrow heads indicate the reducing terminals of the chondroitin sulphate chains.

Observation of the course of reaction with hyaluronidase may yield information of another sort. Thus if each polysaccharide chain were combined at several points to a protein core, then prolonged action of hyaluronidase would be expected to break down the loops so formed and the product would have a higher reducing power than the starting material. In Fig. 1 this situation is depicted; the heavy line represents the protein core and the arrowheads represent the terminal reducing residues of the polysaccharide chains. It is clear, however, that an experiment of this sort will be ambiguous unless there is assurance that fragments of polysaccharide chains not attached to protein have been removed from the system. To this end a membrane of very high permeability was employed for ultrafiltration (for details of the membrane see the Methods section), and comparative experiments were carried out in which the degree of polysaccharide-chain breakage was varied by varying the time of digestion with hyaluronidase and the amount of enzyme. A series of experiments of this kind should result in end products which approach constant reducing power when no free chains remain of a size sufficient to retain them in the sac used for ultrafiltration. An analysis of the final experiments of the series appears in Table 3. It will be observed that with preparation 1 (incubation time 24 hr.) the mixture in solution after treatment with hyaluronidase had an average polysaccharide chain length of 4500 whereas with preparation 2 (incubation time 72 hr.) this average was 2450. In each ultrafiltration experiment the total glucose equivalent of the solution to be ultrafiltered was recovered nearly quantitatively in the ultrafiltrate and in the solution remaining in the sac. In the first experiment the chondroitin sulphate–protein complex taken had 25 ± 2 reducing terminals/10^6 g. of complex, whereas the 'core material' remaining in the sac had 41 ± 2 terminals/10^6 g. of starting material. With preparation 2 the chondroitin sulphate–protein complex (a different extract) had 19 ± 2 reducing residues/10^6 g. and the core substance remaining in the sac had 23 ± 2 reducing residues, calculated on the same weight of initial material. The results of the series showed that the values

Table 3. *Ultrafiltration of hyaluronidase-treated complex*

	Preparation 1 (incubated for 24 hr.)	Preparation 2 (incubated for 72 hr.)
(A) Original chondroitin sulphate–protein complex		
Dry weight (mg.)	195	251
Ash (% as Na_2SO_4)	—	22·7
Nitrogen (%)	5·25	5·72
Total glucose equivalent (mg.)	0·86	0·86
Cn	40 600	53 000
Number of reducing terminal residues in 10^6 g. of complex	25±2	19±2
(B) Chondroitin sulphate–protein complex after treatment with hyaluronidase		
Dry weight (mg.)	195	251
Total glucose equivalent (mg.)	7·84	18·1
Cn	4 500	2 450
(C) In ultrafiltrate		
Dry weight (mg.)	100·2	208
Ash (%)	—	28·0
Nitrogen (%)	2·78	2·93
Total glucose equivalent (mg.)	6·32	17·44
Cn	4 530	2 200
(D) In sac		
Dry weight (mg.)	107·7	44·6
Ash (%)	11·19	8·35
Nitrogen (%)	8·85	9·85
Total glucose equivalent (mg.)	1·44	1·04
Cn	13 500	7 730
Number of terminal residues in 10^6 g. original wt. of complex	41±2	23±2

approached a 1:1 ratio as the degree of degradation increased. It is therefore concluded that each polysaccharide chain is attached to the protein core by a single linkage.

DISCUSSION

Chondroitin sulphate is now regarded as a linear polymer of the substituted disaccharide *N*-acetylchondrosine sulphate. Crystalline derivatives of chondrosine were isolated from the acid-hydrolysis products of chondroitin sulphate by Hobting (1914) and by Levene (1941), and structures for the disaccharide, differing in detail, were formulated by other workers (Meyer, Odier & Siegrist, 1948; Wolfrom, Madison & Cron, 1952; Meyer & Baldin, 1953). Davidson & Meyer (1954), in agreement with Masamune, Hiyama & Kobayasi (1942), offered the first conclusive evidence for the constitution of chondrosine as 3-*O*-(β-D-glucopyranosiduronic acid)-2-amino-2-deoxy-D-galactose.

Early methylation studies (Bray, Gregory & Stacey, 1944) suggested that the polysaccharide may be highly branched, but the isolation of chondrosine in high yield (60–70%) from the acid-hydrolysis products of chondroitin sulphate (Wolfrom *et al.* 1952; Davidson & Meyer, 1954) shows that glycosidically-linked branch chains cannot be present in large amount. The polysaccharide contains alternate hexosaminidic and uronidic bonds and both bovine testicular hyaluronidase and dilute acids degrade it by hydrolysing the hexosaminidic links (Rapport, Meyer & Linker, 1951). In chondroitin sulphate A the sulphate ester residue is linked to galactosamine through position C-4, but bovine nasal-septum cartilage also contains a small amount of the isomeric chondroitin sulphate C, which contains C-6 galactosamine-linked sulphate (Mathews, 1958; Hoffman Linker & Meyer, 1958).

Chondroitin sulphate exists in hyaline cartilage as a protein-containing complex of high molecular weight, but even when extracted under mild conditions the product appears to be polydisperse in molecular size. Webber & Bayley (1956) gave values for the molecular weight of the complex, as estimated by sedimentation and viscosity measurements, of about $1{\cdot}0 \times 10^6$, and Bernadi (1957) reports $1{\cdot}98 \times 10^6$ from viscosity, sedimentation and light-scattering. Webber & Bayley (1956) studied the product obtained from the chondroitin sulphate–protein complex after tryptic digestion of the protein moiety and reported that the number-average molecular weight of the polysaccharide residue was 39 000 (sedimentation and viscosity), but a value of 145 000 was found for the weight-

average molecular weight from light-scattering data. Their product was accordingly regarded as widely polydisperse.

A different conclusion was reached by Mathews (1956), who isolated a chondroitin sulphate–protein complex from an extract of bovine hyaline cartilage and after treating it with proteolytic enzymes found that the product behaved as a monodisperse solution with molecular weight 50 000. Later Mathews & Lozaityte (1958) from light-scattering and viscosity data proposed that the basic molecular unit of the chondroitin sulphate–protein complex is a rod of length 3700 Å and $4{\cdot}0 \times 10^6$ molecular weight. To account for the linear dimensions and distribution of matter in the particle they proposed that the protein moiety forms a core running the length of the rod, along which is distributed about 62 units of chondroitin sulphate, each of 50 000 molecular weight. Evidence was also presented which suggests the possibility that aggregates of much higher molecular weight may be formed by both lateral and end-to-end association of the basic units and that additional protein may be involved in this process.

In the present work the problem has been approached in another way. Since it is known that there are few, if any, glycosidically-linked side chains in the chondroitin sulphate of hyaline cartilage, it was thought that the observed formation of very large molecules was probably through links or branching points which do not involve the reducing group of the terminal galactosamine residue. Provided that the terminal residue is not masked in this or any other way, the number-average weight of linear chondroitin sulphate chains making up a multiple-chain molecule can be determined by measurement of the reducing power. The result can then be compared directly with the number-average molecular weight of the whole molecule, determined osmotically, to give the number of chains in the molecule.

The chain weight (*Cn*) of the complex extracted by water was $40–50 \times 10^3$ in different preparations. If this value is corrected for the non-chondroitin sulphate content of the complex (about 25 %), the true polysaccharide chain weight becomes $30–40 \times 10^3$. Osmotic measurements carried out with the chondroitin sulphate–protein complex give pressures corresponding to a molecular weight in the range $1–5 \times 10^6$. This estimate could not be obtained with greater precision since with polyions of such high molecular weight the Donnan correction for pressure due to the counter ions is a very high proportion of the total osmotic pressure. The two results together suggest that, as a minimum, the particles of chondroitin sulphate–protein complex in solution contain 20 chondroitin sulphate chains.

A similar result was obtained in another way. After removal of the protein from the complex by treatment with papain, the osmotic molecular weight of the preparation fell to 28 500, with a possible error of ± 1000, showing that the papain treatment had released at least 30 chondroitin sulphate chains from combination in the molecule. The value for the osmotic molecular weight of the chondroitin sulphate released lies rather below that obtained for the corrected chain weight determined for the original molecule, and suggests that a few of the polysaccharide chains had masked terminal residues.

When the complex was treated with hyaluronidase, sections of the polysaccharide chains were split off, leaving a protein-containing core apparently intact. Short-chain fragments were separated from the core by ultrafiltration through a membrane of high permeability and, in experiments where degradation with the enzymes proceeded far enough, separation of the core from chondroitin sulphate chain fragments could be taken to completion by final dialysis. In a series of experiments on these lines the reducing power of the core material remaining in the membrane sac approached a constant value that was close to the reducing power of the original complex. This indicates that each polysaccharide chain in the chondroitin sulphate–protein complex is attached to the protein core at one point or one small region only. The osmotic molecular weight of the core material, which contained about 30 % of chondroitin sulphate, was 170 000; this leads to the value 120 000 for the non-chondroitin sulphate material of the core. It is shown in the next paper that this material is a mucoprotein containing a considerable amount of neutral sugar and hexosamine.

Fig. 2. Model proposed for the structure of the chondroitin sulphate–protein complex. The protein core (heavy line) carries at least 23 chondroitin sulphate chains of *Cn* 28×10^3. These are connected by a single linkage which does not involve the terminal reducing residue (indicated by arrow heads). The core also carries, at some point along its length, a second polysaccharide material (indicated by a circle) which is rich in galactose and glucosamine.

The model which emerges is depicted in Fig. 2. In this, the particle of minimum molecular weight is conceived as a single protein chain (the heavy line), along which are distributed a large number of chondroitin sulphate chains each attached at a single point, the majority bearing a reducing terminal residue (represented by an arrow head). The protein chain also carries, at some point or points along its length, further polysaccharide material (represented by a circle) which contains neutral sugars and hexosamine.

The work reported here thus essentially confirms and extends the proposal by Mathews & Lozaityte (1958), although the values for the molecular weight of the protein-free chondroitin sulphate chains are considerably lower than those given by these authors and by Mathews (1956) and Mathews & Dorfman (1953). The molecular dimensions of the basic unit of the chondroitin sulphate–protein complex must remain in doubt because of the heterogeneity of its solutions and the possible formation of aggregates.

Determinations of the reducing power of the core material given in this paper showed that the core contained 23 reducing chains, each consisting, as an average value, of about 4–5 disaccharide repeating units. If the mucoprotein core, together with its 23 reducing chains, represents a residue of the smallest molecular unit of the chondroitin sulphate–protein complex, the molecular weight of the whole unit would be about 750 000 and the particles in solution with observed molecular weights of $1–5 \times 10^6$ must be regarded as aggregates. However, it is possible that labile bonds in the mucoprotein core are split during the course of treatment with hyaluronidase and that the true molecular weight of the complex is accordingly higher.

SUMMARY

1. The polysaccharide chain weight (*Cn*), estimated by measuring the reducing power of the terminal residue, was compared with the osmotic molecular weight (*Mn*) for chondroitin sulphate–protein complexes extracted from bovine hyaline cartilage.

2. Water extracts prepared from fresh cartilage slices in a high-speed homogenizer yielded a chondroitin sulphate–protein complex containing approx. 75 % of chondroitin sulphate having *Mn* $1–5 \times 10^6$ and *Cn* 50×10^3. Thus the 'osmotic molecule' contained 20–100 chondroitin sulphate chains terminating in reducing residues.

3. Removal of protein by digestion with papain decreased *Mn* to $28 \times 10^3 \pm 2000$ and *Cn* remained unchanged. The result indicates that the chondroitin sulphate chains in the complex are linked together by protein and that the reducing terminals of a proportion of the chondroitin sulphate chains freed by papain are masked.

4. When the native complex was treated with ovine hyaluronidase and the chondroitin sulphate chain fragments were removed by ultrafiltration, the chondroitin sulphate content of the remaining core was decreased to about 30 % and *Cn* was 7730. The protein-rich core had *Mn* 170×10^3, showing the presence of 23 chondroitin sulphate chains of decreased length.

5. The complex had approximately the same reducing power before as after treatment with hyaluronidase and ultrafiltration, thus showing that each chondroitin sulphate chain is attached to protein at one point only.

6. It is concluded that the complex may exist in solution as a macromolecule of *Mn* $1–5 \times 10^6$ or an aggregate of smaller molecular units of *Mn* at least 750×10^3. The units contain at least 23 chondroitin sulphate chains of *Cn* 28×10^3. These are connected to a protein core by a single linkage which does not involve the terminal reducing residue.

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The Chemistry of Connective Tissues

7. DISSOCIATION OF THE CHONDROITIN SULPHATE–PROTEIN COMPLEX OF CARTILAGE WITH ALKALI

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In the preceding paper a model was proposed for the constitution of the chondroitin sulphate–protein complex of cartilage in which at least 23 chondroitin sulphate chains are connected to a protein core by single linkages thought to be of covalent character. In the work now described, the dissociation of the complex in alkaline solution was studied more closely. Electrophoresis experiments in columns stabilized by a density gradient suggested that alkaline dissociation is irreversible and follows a time course related to the hydroxyl ion concentration. The protein core split off was isolated and analysed and was found to carry a second amino polysaccharide containing glucosamine and galactose.

METHODS

Chondroitin sulphate–protein complex. The complex was prepared from dried cartilage powder as described in the preceding paper. As it was important to decrease contamination with collagen to the lowest possible extent, treatment with ion-exchange resin (Amberlite CG 50) was repeated three or four times until the hydroxyproline content was less than 0·2% as judged by ion-exchange chromatography of the hydrolysed product.

Zone electrophoresis. The experiments were carried out with an electrophoresis column stabilized by a sucrose density gradient. The method used was generally similar to that described by Svensson, Hagdahl & Lerner (1957), but since the design of the apparatus was different, and has proved to be advantageous in some respects, a drawing of it (Fig. 1) is given here. The apparatus was symmetrical and consisted of two similar glass columns, A and A′, provided with ball-joints and arranged over a U tube B. Duplicate sets of columns with different diameters were provided so that the apparatus could be adjusted to suit the scale of the separation. The electrical circuit was completed by the provision of two vessels, C and C′ (diameter 7·5 cm.), containing large silver–silver chloride electrodes and connected through large-bore taps, D and D′. A bridge E was provided to secure hydrostatic equilibrium when the taps D and D′ were closed.

In the experiment described the columns were 2·7 cm. in diameter and the height between H and J′ was 34 cm. The columns were not provided with water jackets and the experiment was carried out at room temperature.

The apparatus was first filled with buffer (0·005 N-NaOH containing 0·015 N-NaCl) to a point above the level of the side arms D and D′. A reservoir filled with dense buffer (600 ml., 0·01 N-NaOH containing 0·025 N-NaCl and 50%, w/v, sucrose) was connected in series with a mixing chamber (capacity 550 ml.) and a metering pump. The mixing chamber was provided with a magnetic stirrer. The buffer stream of increasing density was pumped from the mixing chamber into the U tube through the tap I. The

flow rate was 10 ml./min. and pumping was continued until sufficient buffer had passed to displace the volume in the U tube to about 4 cm. above the level of the inlet tubes J and J′.

Tap D′ was then opened and D closed. Tap G′ was opened slowly to allow the collection of a few millilitres of buffer from the jet. This caused a sharp step in the density gradient at the point J′. The operation was then repeated on the opposite side with D′ closed and D open, collecting from the jet at G. Before introduction of the sample, D′ and D were both closed and F′ and F both opened to allow hydrostatic equilibration via the bridge.

The sample (52 mg.) was prepared as a solution (0·5%, w/v) in the buffer used to fill the apparatus, and was adjusted by the addition of a few drops of the sucrose buffer until its refractive index was the same as that of the mixed sample from the gradient which had been drawn out of the jet. This ensured that the sample was of the correct density to remain suspended in the interface at point J′. The sample was then drawn in through the inlet tube G and injected into the column at J′, by closing taps D and D′ and slowly opening tap I. To balance the column the same volume of buffer was drawn off from tap I with tap D opened and tap D′ closed. After closing tap D, saturated NaCl solution was introduced into the electrode vessels through funnels K and K′ to cover the exposed metal of the silver electrodes (L and L′) and the apparatus was again equilibrated by opening the bridge. This was closed before applying the current.

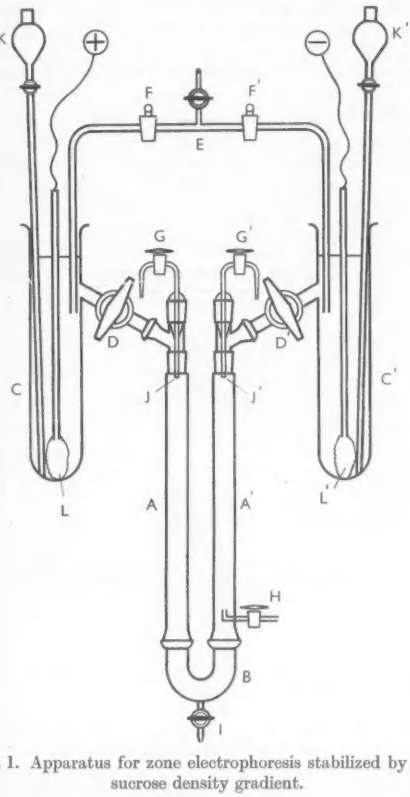

Fig. 1. Apparatus for zone electrophoresis stabilized by a sucrose density gradient.

The apparatus was allowed to stand for about 30 min. with taps D and D′ open to bring it into temperature equilibrium and to allow some diffusion of the lower edge of the zone. A current of 17 mA (370 v) was then applied and migration was allowed to proceed for 17 hr. Fractions (4 ml.) were then run off by hand into graduated tubes, buffer from vessel C being allowed to replace the solution in the column. The fractions were analysed for protein by the method of Lowry, Rosebrough, Farr & Randall (1951), with a solution of gelatin as standard, and for hexosamine by the method of Rondle & Morgan (1955), after hydrolysis with 4 N-HCl for 16 hr. in sealed tubes at 100°.

Chromatographic fractionation of the core protein separated by treatment with alkali. The column (2·5 cm. × 15 cm.) was packed with the carboxylic acid resin Amberlite CG 50. The resin was passed through a sieve (200 mesh/in.) and prepared for use as described by Boardman & Partridge (1955). A freeze-dried preparation of the chondroitin sulphate–protein complex (2 g.) was dissolved in 200 ml. of 0·5 N-KOH and allowed to stand at 25° for 20 hr. After neutralizing with acetic acid and dialysing against distilled water the solution was made up to 340 ml., potassium acetate being added to bring the concentration to 0·1 M and acetic acid to pH 4·9.

The column was equilibrated to pH 4·9 by washing for 24 hr. with the acetate buffer, and the solution of the alkali-treated complex was then applied at 20 ml./hr., the effluent being collected as a single fraction. The column was provided with a buffer reservoir connected in series with a magnetically-stirred mixing chamber (500 ml. capacity). The reservoir and mixing chamber were filled with 0·25 M-potassium acetate buffer, pH 4·9, and 140 ml. of this solution was passed through the column at 20 ml./hr., the effluent being collected in 20 ml. fractions. The solution in the reservoir was replaced with 1 M-potassium acetate buffer of the same pH for 14 fractions and was then replaced with 0·4 M-Na_2HPO_4. The fractions were estimated for protein by the method of Lowry *et al.* (1951), with a solution of gelatin as standard (Fig. 3).

Qualitative analysis of the neutral sugars in the core material. Fractions from the core protein (15 mg.) were dissolved in 1·2 ml. of 0·5 N-H_2SO_4, sealed in a glass tube and hydrolysed at 100° for 4 hr. The solution was then neutralized to pH 5 with $Ba(OH)_2$ solution and the precipitated barium sulphate removed by centrifuging. The clear solution and washings were passed through a small column (100 mg.) of sulphonated polystyrene resin (Dowex-50) in its H^+ form, to remove amino acids and peptides. The column effluent and washings were concentrated by evaporation under reduced pressure and freeze-dried.

The product was then dissolved in 0·05 ml. of water. Single-dimensional paper chromatograms were prepared with butanol–acetic acid–water (Partridge, 1948) and ethyl acetate–pyridine–water (Jermyn & Isherwood, 1949). Aniline phthalate (Partridge, 1949) and naphtharesorcinol (Partridge, 1948) were used as spraying reagents for revealing the spots.

Estimation of hexosamines, hydroxyproline and reducing power. The methods used were those reported by Partridge *et al.* (1961).

Amino acid analysis. The chromatographic procedure was that of Moore & Stein (1951). For colour development the ninhydrin reagent of Yemm & Cocking (1955) was used.

RESULTS

Dissociation of the chondroitin sulphate–protein complex

It has already been reported (Partridge & Davis, 1958) that on zone electrophoresis (starch column) at pH 8·54 the chondroitin sulphate–protein complex migrates substantially as a single zone. Brief treatment with NaOH solution did not affect the stability of the complex in the electric field, but after treatment with 0·5 N-NaOH for a fairly prolonged period the complex dissociated and on zone electrophoresis under the same conditions the material migrated as two zones, one consisting of protein and the other of protein-free chondroitin sulphate. The results of this and other similar experiments (cf. Muir, 1958) have been taken to indicate that the polysaccharide and protein in the complex are combined by primary valency. This, however, is not necessarily true since the complex may dissociate instantaneously in alkaline solution and this dissociation may be reversed when the solution is dialysed or neutralized in preparation for electrophoresis or ion-exchange treatment. This situation occurs not infrequently in investigations of protein–polysaccharide complexes and could be resolved if a separation technique were available which could be applied to highly alkaline solutions.

It was thought that zone electrophoresis, with a density gradient to stabilize against convection (Svensson *et al.* 1957), might provide such a technique. Accordingly this method was attempted with sucrose to provide the density gradient and 0·01–0·02 M-NaOH as the electrolyte. Electrophoresis columns of this sort proved to be unsatisfactory owing to marked polarization which developed a few minutes after application of the current. In sucrose–NaOH systems the OH^- ion concentration is markedly decreased owing to the formation of sucrose anions, and a situation results in which the transport number of the cations (Na^+) is very much higher than that of the anions (sucrose$^-$ and OH^-). This results in the rapid depletion of Na^+ ions at the sucrose interface in the anode limb. (For theoretical discussion of concentration polarization of this type see Partridge & Peers, 1958.) It was found that polarization could be much decreased by the addition of a neutral salt to the solution, and accordingly the experiments described here have been performed with a mixture of NaOH (0·005–0·01 M) and NaCl (0·015–0·025 M).

Fig. 2 (*a*) shows the pattern obtained by electrophoresis of the chondroitin sulphate–protein complex at 370 V for 17 hr. This should be compared with Fig. 2 (*b*), which shows the pattern obtained under as nearly as possible identical conditions by electrophoresis of the complex after treating it with 0·5 N-NaOH for 20 hr. at 25°. In both cases 25 fractions each of 4 ml. were taken after completion of the run and samples from each were estimated for protein by the method of Lowry *et al.* (1951), and for chondroitin sulphate by measuring galactosamine after hydrolysis with 4 N-HCl for 20 hr.

Fig. 2 (*a*) shows that the native complex migrated substantially as a single zone in dilute NaOH solution. However, the position of the protein and chondroitin sulphate peaks were skewed with respect to each other, indicating some degree of heterogeneity. This may have been due to partial hydrolysis during the electrophoresis experiment, or it may indicate the presence of a small amount of protein impurity in the original preparation. As will be shown later, contamination with a large amount of a second protein is unlikely, since the isolated protein component appears to be substantially homogeneous in amino acid composition.

The electrophoretic pattern obtained after a preliminary hydrolytic treatment with NaOH (Fig. 2*b*) shows the almost complete separation of

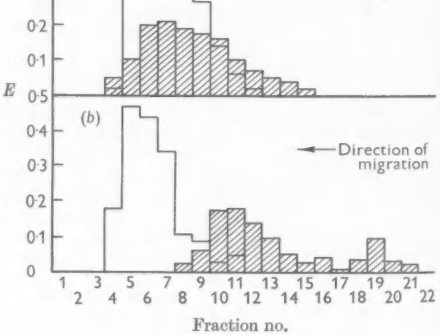

Fig. 2. Zone-electrophoresis pattern given by the chondroitin sulphate–protein complex at 370 V for 17 hr. in NaOH–sucrose–NaCl buffer. (*a*) Pattern given by the native complex. (*b*) Pattern given by the complex after treatment with 0·5 N-NaOH for 20 hr. at 25°. Hatched blocks, protein (Folin reagent). Open blocks, chondroitin sulphate (Elson & Morgan reagent).

protein from chondroitin sulphate and demonstrates the irreversible nature of the dissociation of the two components of the complex by the prolonged action of alkali (20 hr.). Pretreatment with 0·5 N-NaOH solution for shorter periods (8 or 16 hr.) provided only partial separation of the protein and carbohydrate components. In Fig. 2 (*b*) the zone due to protein shows considerable spread and the substance is clearly not homogeneous in charge density. This suggests that the protein is degraded to some extent; however, such degradation would be expected since it has been shown that similar treatment results in the hydrolysis of labile peptide bonds in proteins such as collagen (Bowes & Kenten, 1948) or gelatin (Saunders & Ward, 1955).

Estimation of the polysaccharide chain weight (*Cn*) by titration of the reducing end groups (Partridge, Davis & Adair, 1961) showed that *Cn* for the untreated complex was 46 800 and for the product of alkaline hydrolysis 24 100. After dialysis in Visking tubing, *Cn* for the alkali-treated product rose to 30 600. This indicated that the chondroitin sulphate moiety also suffered some degradation by chain rupture during alkaline treatment.

Homogeneity of the protein component

Thus far no means have been found whereby the protein component may be isolated under conditions that would be unlikely to cause secondary changes in the protein itself. The use of alkali, under as mild conditions as possible, is effective in separating chondroitin sulphate from the protein; but these same conditions can be expected to damage the protein by the rupture of easily hydrolysable peptide bonds or by liberation of ammonia from asparaginyl or glutamyl residues. The homogeneity of the protein prepared by alkaline hydrolysis was examined, and since only small amounts of the material were available a chromatographic procedure, with an ion-exchange-resin column, was adopted. Details of the procedure are given in the Methods section.

Fig. 3 shows the chromatogram obtained from the product of degrading the chondroitin sulphate–protein complex with 0·5 N-KOH for 20 hr. at 25°. The fractions from peak A were collected together and the product was recovered by dialysis followed by drying from the frozen state. The composition of the product [N, 2·8 %; galactosamine, 27·5 %; ash (as K_2SO_4), 30·1 %] showed it to be potassium chondroitin sulphate substantially free from protein. The protein moiety was not eluted at pH 4·9 at any concentration of salt up to 1 M, but when a pH gradient was provided by supplying Na_2HPO_4 to the mixing chamber it appeared as a series of partly resolved peaks (Fig. 3), which fell into two broad areas (labelled P 1 and P 2 in the Figure). The fractions grouped under P 1 and P 2 were isolated separately for analysis and Table 1 shows comparative analytical data for the two preparations.

The absence of hydroxyproline in both substances showed that neither was derived from collagen. Both materials showed similar u.v.-absorption curves (Fig. 4), and when the ratio of tyrosine to tryptophan was calculated from the equations of Goodwin & Morton (1946) the results for the two fractions were closely similar. Both materials contained substantial amounts of neutral sugar and the ratio of glucosamine to galactos-

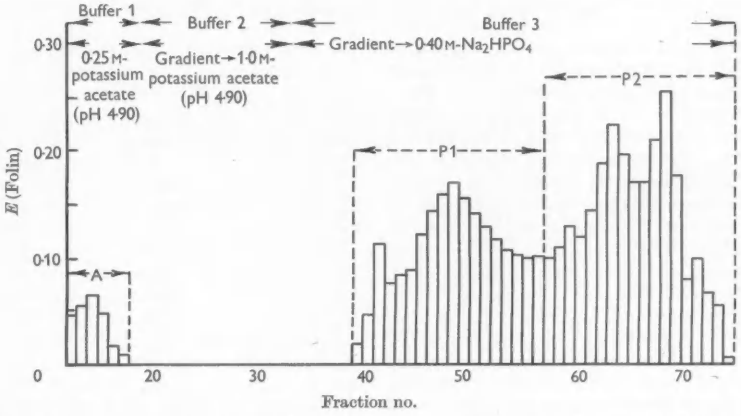

Fig. 3. Chromatographic fractionation of the core protein separated by treatment with alkali. Column 2·5 cm. × 15 cm.; resin Amberlite CG 50. The fractions were estimated for protein with the Folin reagent (Lowry, Rosebrough, Farr & Randall, 1951).

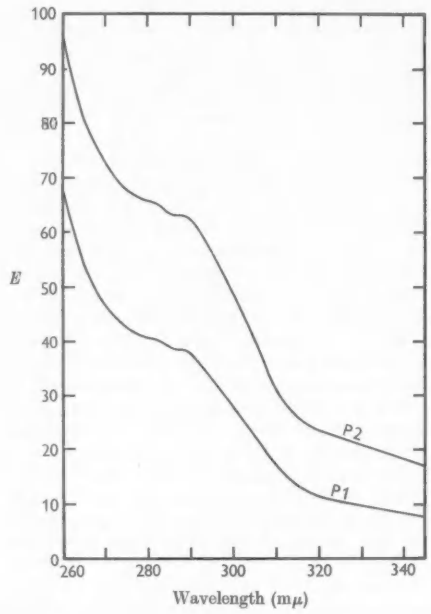

Fig. 4. Extinction (u.v.) of the head and tail fractions from the core protein separated by ion-exchange chromatography. Solvent, 0·1 N-NaOH. P1, 0·39 mg./ml. (N, 10·7%); P2, 0·41 mg./ml. (N, 11·81%).

amine, as measured by ion-exchange chromatography after acid hydrolysis, was similar in the two fractions. Table 2 shows the amino acid composition of fractions P1 and P2, estimated by ion-exchange chromatography of the products of acid hydrolysis. Whereas the results for a number of the amino acids show differences outside the normal range of error for the method, the pattern of amino acid distribution is similar for the two fractions. Comparison of the analytical results for the two fractions given in Tables 1 and 2 suggests that the material isolated by alkaline degradation has been derived from a single molecular species containing both protein and polysaccharide and that degradation has resulted in partial removal of

Table 1. *Analytical data for the head and tail fractions from the protein core after separation by ion-exchange chromatography*

	Fraction P1	Fraction P2
Weight of fraction (mg.)	62·6	40·0
Nitrogen (%)	10·66	11·81
Hydroxyproline (%)	Nil	<0·2
Tyrosine/tryptophan*	1·7	1·9
Neutral sugars (%) as glucose (anthrone)	6·1	3·5
Glucosamine (%)	2·5	1·24
Galactosamine (%)	0·54	0·44
Uronic acid (%) as glucuronic acid (carbazole)	0·48	0·24

* Moles of tyrosine and tryptophan in 1 g. of protein, determined from E at 280 and 294 mμ and the equations of Goodwin & Morton (1946).

Table 2. *Amino acid composition of the head and tail fractions resulting from chromatographic fractionation of the core protein*

	Head fraction P1		Tail fraction P2	
	Amino acid N (% of total N)	g. of amino acid/ 100 g. of dry ash-free sample	Amino acid N (% of total N)	g. of amino acid/ 100 g. of dry ash-free sample
Nitrogen	—	10·66	—	11·81
Aspartic acid	4·59	4·55	5·83	6·54
Threonine	2·10	1·90	2·51	2·52
Serine	2·51	2·00	3·34	3·00
Glutamic acid	9·26	10·4	7·30	9·08
Proline	8·87	7·78	5·20	5·10
Glycine	10·60	6·04	7·92	5·00
Alanine	4·88	3·31	4·60	3·40
Valine	3·18	2·83	4·30	4·20
Methionine	0·65	0·74	0·94	1·20
Isoleucine	2·32	2·31	4·50	3·10
Leucine	4·65	4·64	6·50	7·20
Tyrosine	2·83	3·90	2·30	3·50
Phenylalanine	1·41	1·77	2·80	3·95
Lysine	3·24	1·81	5·90	3·63
Histidine	2·57	1·01	4·00	1·73
Arginine	10·85	3·60	14·5	5·30
Hydroxyproline	Nil	Nil	<0·2	<0·2

polysaccharide and some loss of small peptides. Loss of carbohydrate was greater for the slower-running fractions grouped under P 2.

Composition of the carbohydrate portion of the core material

Fractions P 1 and P 2 were hydrolysed with dilute H_2SO_4 for identification of the neutral sugars by paper chromatography. After removal of amino acids, peptides and amino sugars with sulphonated polystyrene resin, chromatograms were prepared with butanol–acetic acid–water and pyridine–ethyl acetate–water. Both P 1 and P 2 gave heavy spots for galactose but traces only of glucose and mannose. The polysaccharide moiety of the core protein thus contained glucosamine and galactose in relatively large amounts together with smaller quantities of galactosamine and uronic acid. This suggests the presence of keratosulphate (Meyer, Linker, Davidson & Weissmann, 1953) as the major alkali-stable component, together with a small amount of galactosamine and uronic acid as a residue of the chondroitin sulphate chains split off by the action of alkali.

DISCUSSION

Warner & Schubert (1958), using the boundary-electrophoresis method of Tiselius, showed that the chondroitin sulphate–protein complex of bovine nasal cartilage migrated as a single peak at all values over the range pH 2–10. At pH 11 a very small component with a lower mobility appeared but in sodium hydroxide solution of pH 12·5 the boundary split into two parts, the slower-moving part representing about 30 % of the pattern area. The dissociation was not reversible since when the solution exposed to pH 12·5 was subsequently examined at pH 3·3 the two components were still evident. Analysis results for the separated components were not available, but Warner & Schubert (1958) pointed out that the high negative mobility of the smaller component showed that it could not be purely protein in character and that exposure to alkali under these conditions did not result in a clean split between protein and polysaccharide. It appears rather that the slower-moving component contained the protein part of the complex with some sulphated polysaccharide still attached.

In the zone-electrophoresis experiments we report here the hydroxyl ion concentration of the buffer solution was less than that of a sodium hydroxide solution of the same concentration, owing to the presence of sucrose, and values obtained with the glass electrode were near pH 10·8. Substantial degradation by alkali during the course of the run, as reported by Warner & Schubert (1958), would therefore not be expected. It was found that, as minimum conditions, treatment with 0·5 N-sodium hydroxide for 20 hr. at 25° was necessary in order to produce complete hydrolysis. Under these conditions the dissociation was irreversible, and pure chondroitin sulphate and a protein fraction could be isolated readily from the product by zone electrophoresis. The protein fraction prepared in this way contained up to 0·5 % of galactosamine and 0·5 % of uronic acid, suggesting that even after the severe alkaline treatment it still contained chondroitin sulphate to the extent of 1–2 %. The results in the preceding paper show that in the native chondroitin sulphate–protein complex the chondroitin sulphate chains are linked to a protein core at a single point or small area, and taking this information together with the present demonstration of the irreversible nature of the alkaline dissociation, the evidence strongly suggests that the polysaccharide chains are combined to protein by covalent bonds which are fairly readily hydrolysed by alkali. Attempts are now being made to determine the nature of these bonds.

The high degree of spreading of the protein zone during electrophoresis (Fig. 2*b*) suggests that the protein liberated by alkaline treatment is heterogeneous at least as regards charge density, and this was confirmed by chromatographic fractionation with an ion-exchange column (Fig. 3). In the column experiment the protein was eluted as a group of poorly resolved peaks, suggesting a degraded product. Head and tail fractions were analysed separately and it was found that both fractions were free from hydroxyproline, showing the absence of collagen-degradation products: both fractions had similar ratios of tyrosine to tryptophan and similar amino acid analysis, showing that they were most probably derived from a single native protein. However, the content of carbohydrate in the two fractions, though of the same composition, varied considerably in amount.

The analytical results as a whole suggest that the protein fractions were derived from an original polysaccharide-bearing protein contained in the core of chondroitin sulphate–protein complex, and that the variations in polysaccharide content and net charge were due to varying degrees of degradation suffered during the treatment with alkali used to dissociate the complex. Both protein fractions, besides containing a small amount of galactosamine and uronic acid, which may represent a remaining trace of chondroitin sulphate, also contained considerable amounts of glucosamine and galactose. This result is strongly suggestive of the presence of keratosulphate and it is possible that the chondroitin sulphate–protein complex contains keratosulphate bound to the protein core by links that are more resistant to alkali than those binding chondroitin sulphate.

There remains the possibility that keratosulphate is present as an independent protein complex and that the initial material is a mixture. From the results given this seems unlikely. The ratio of galactosamine to glucosamine in the original complex was 6:1. After alkaline hydrolysis this ratio in the protein moiety was 1:5 in the head fraction and 1:3 in the tail fraction and nearly one-third of the original glucosamine was recovered with the protein. If keratosulphate were present as an independent complex originally this must be much less readily hydrolysed by alkali than is the chondroitin sulphate–protein complex. It would therefore be retained as a highly charged anion and would easily be separable from protein both by electrophoresis and by ion-exchange chromatography. This was not found; in the ion-exchange chromatogram there was wide separation between chondroitin sulphate and protein, but the glucosamine appeared with the protein peak and its concentration was not very different in head and tail fractions.

A conclusive decision on this point is not easy to obtain in a study of the present kind owing to the difficulty in separating large anionic protein–polysaccharide complexes, which may form heterogeneous aggregates. A more revealing approach to the problem may lie in an attempt to isolate the protein moiety by a method which does not give rise to extensive degradation, and work is now in progress on these lines.

SUMMARY

1. The dissociation of the chondroitin sulphate–protein complex of hyaline cartilage in alkaline solution was studied by zone electrophoresis in a column stabilized by a density gradient.

2. The complex was stable in a sodium hydroxide–sucrose solution of pH 10·8 but was irreversibly dissociated by treatment with 0·5 N-sodium hydroxide for 24 hr. at 25°. It was concluded that linkage between chondroitin sulphate and protein is most probably covalent.

3. The protein fraction released by alkaline treatment was examined by chromatography with a carboxylic acid ion-exchange resin (IRC-50).

4. The protein was heterogeneous and appeared as a close group of poorly resolved peaks. Head and tail fractions were analysed separately and found to have the same qualitative composition, but the head fraction contained 9·6 % of carbohydrate and the tail fraction 5·4 %. It was concluded that the fractions were derived from a single carbohydrate-containing protein which had been degraded by the action of alkali.

5. The carbohydrate moiety of the protein was rich in glucosamine and galactose, but neutral sugars other than galactose were present in trace amount only. The core protein may thus contain keratosulphate.

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A Study on Globulin Formation by Plasma-Cell Neoplasm (5563) Transplantable in Mice

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Antibody and γ-globulin are formed by a number of tissues with highly heterogeneous cell populations such as bone marrow, lymph glands and spleen. Only a small part of the cells in these tissues are plasma-type cells in which antibody protein and at least some of the γ-globulin normally found in serum originate. This makes a study of globulin synthesis difficult. Recently, Dr M. Potter and his collaborators at the National Cancer Institute, Bethesda, have established several lines of plasma-cell tumours transplantable in inbred strains of mice. Each plasma-cell neoplasm is associated with different but characteristic globulins in high concentrations in the serum (Potter & Fahey, 1960). On electrophoresis of the serum at pH 8·6 these globulins migrate in the region of serum γ- and β-globulin. The condition in mice shows great similarity to the human malignant disease, multiple myeloma. The tumour cells are basophilic cells with vacuoles and acentric nuclei characteristic of plasma cells normally found in lymphoid tissue (Dunn, Potter, Fahey & Merwin, 1960). Electron micrographs of thin sections show that the cell cytoplasm has a highly organized endoplasmic reticulum (A. J. Dalton, unpublished work) similar to that found in hepatic or pancreatic cells and normal plasma cells. This organization is characteristic of cells active in protein formation and secretion.

The subcutaneous mouse tumours provide tissue to study the synthesis of globulin *in vitro* and make it possible to follow globulin formation by plasma cells over many transplant generations. One of the plasma-cell neoplasms (5563) has been studied in detail, since the globulin in the serum of animals bearing the 5563 tumour was found to be very similar to normal mouse γ-globulin in its immunological properties. It is referred to below as γ-globulin or γ-myeloma protein. Dr B. Balfour at this Institute stained sections of the 5563 tumour with fluorescein-labelled rabbit antibody against purified myeloma protein. Almost all cells showed a highly fluorescent cytoplasm and therefore contained myeloma protein. The tumour tissue thus provides us with a fairly homogeneous population of plasma cells. In the work described in this paper the protein products were analysed by starch-gel electrophoresis and were found to be heterogeneous and to consist of at least five components. These stayed constant over many transplant generations.

The origin of serum-myeloma proteins has not been certain. Nathans, Fahey & Potter (1958) have studied formation of myeloma protein *in vivo* by injecting radioactive amino acids into mice carrying the 5563 neoplasm. The authors found that the myeloma protein extracted from the tumour had a higher specific radioactivity than the myeloma protein in the serum showing a precursor–product relationship. In the present study it could be shown that slices of tumour tissue form the myeloma protein *in vitro* at a considerable rate. The effect of various amino acid and nucleic acid analogues on synthesis of the globulins and general tissue protein *in vitro* was investigated. Globulin synthesis by subcellular particles was also investigated; although it was possible to obtain a tumour-microsome fraction which incorporated radioactive amino acid into insoluble protein, incorporation into specific globulin could not be demonstrated.

MATERIALS AND METHODS

^{14}C-Labelled amino acids. Biosynthetic [^{14}C]glycine, L-[^{14}C]valine, L-[^{14}C]leucine and algal [^{14}C]protein were obtained from The Radiochemical Centre, Amersham, Bucks., with specific radioactivities of 40, 100, 100 and 250 μc/mg. respectively. The algal protein was hydrolysed overnight at 105° in 6 N-HCl, freed from HCl by evaporation and neutralized; the [^{14}C]amino acids were purified by absorption on a column of Zeo-Karb 225 and subsequent elution with aq. 2 M-NH_3 soln. DL-β-*p*-Fluorophenyl[β-^{14}C]-alanine (2·35 μc/μmole) was obtained from Dr M. H. Richmond, at this Institute.

Nucleic acid analogues. These were mostly obtained from the California Corporation for Biochemical Research. A few substances which were personal gifts will be indicated in the text.

Transplantation of tumours into mice. Breeding pairs of the C_3H/He strain of mice and animals bearing the 5563 plasma-cell neoplasm were sent to us through the kindness of Dr M. Potter, National Cancer Institute, Bethesda. The tumour was transplanted into the F_1 generation by implanting three or four pieces of tissue (about 1 mm. in

size) subcutaneously with a trochar. The tumour developed to a size of 2–4 g. in 6–8 weeks.

Chromatography of myeloma protein (5563 *neoplasm*). The myeloma protein was purified and fractionated by chromatography on diethylaminoethyl (DEAE)-cellulose (Peterson & Sober, 1956) with conditions of gradient elution and packing described by Fahey & Horbett (1959). For purification of myeloma protein, 1 g. of DEAE-cellulose was used/ml. of tumour-bearing mouse serum dialysed against 0·01 M-K_2HPO_4–KH_2PO_4 buffer, pH 6·5. The starting buffer (90 ml.) was put into a cylindrical vessel which was connected by means of a siphon to a conical flask (nominally 50 ml.) containing 55 ml. of more concentrated buffer, 0·2 M-K_2HPO_4–KH_2PO_4, pH 6·5. Mixing in the cylindrical vessel was done with a magnetic stirrer. Fractions (3 ml.) were collected and the protein eluted in the fractions was followed by measuring its extinction at 280 mμ.

The fractions were concentrated by ultrafiltration (Sober, Gutter, Wyckoff & Peterson, 1956) and characterized by electrophoretic and immunological analyses.

Starch-gel electrophoresis. This was carried out by the method of Smithies (1955) with a discontinuous buffer system. Glycine–NaOH buffer, pH 8·9 (0·05 M), was used on the gel. Electrophoresis was continued for 6 hr. at 12 mA for a block of starch gel 25 cm. × 5 cm. × 0·5 cm.

Production of antisera for specific precipitation of myeloma globulin. Rabbits were immunized with 15–20 mg. of 5563 myeloma protein purified by chromatography on anion-exchange cellulose (see Fig. 2). Two injections were given intramuscularly with Freund's adjuvant (Freund & McDermott, 1942) 1 week apart and after 5–6 weeks six intravenous injections of alum-precipitated myeloma protein were administered over 2 weeks. The animals were bled 1 week later.

Incubation of tumour tissue. The tumour was excised after anaesthetizing the mouse with ether and bleeding it by heart puncture. The tumour tissue was rinsed in Hanks's (1948) balanced salt solution and minced with scissors. Known weights of tissue (110–250 mg. wet wt.) were incubated in conical flasks (previously treated with a silicone) in 4 ml. of medium with gentle shaking. The medium was made up of Hanks's solution containing 100 units of streptomycin and penicillin/ml., acid-hydrolysed rabbit globin (5 mg. of N/100 ml.), DL-tryptophan (4 mg./100 ml.) and 0·1 g. of gelatin/100 ml. [^{14}C]Amino acids (1–2 μc) were added to each flask; the flasks were gassed with CO_2/O_2 (5:95) and incubated for various lengths of time. A separate flask was used for each time point.

Isolation of myeloma protein and general tissue protein. After incubation several milligrams of non-radioactive amino acid were added to each flask to minimize adsorption of [^{14}C]amino acids on proteins. The samples were frozen and thawed, homogenized in a Potter-type homogenizer for 1 min. and the sediment was removed by centrifuging for 10 min. at 12 000 *g* at 3°.

The myeloma protein was isolated from the supernatant by specific precipitation with antiserum prepared by immunizing rabbits with purified myeloma protein (as described above). Since it was important to precipitate the myeloma protein with antibody at equivalence point, the globulin content of tumour extract had to be titrated in a control sample (0·1 g. of tumour/ml. of medium extracted in the same way as the incubated tissue). Measured samples of tumour extract were set up in a series of tubes and various amounts of antiserum were added to determine the equivalence point for the antibody–antigen precipitation. (This is the point where all of the antigen is precipitated by the antibody with neither antibody nor antigen present in excess in the supernatant.) Tumour tissue was found to contain 4–5 mg. of myeloma protein/g. wet wt., and the antibody–antigen ratio at the equivalence zone was around 7:1. On this basis the myeloma globulin in the incubation medium was precipitated with a measured sample of the supernatant to yield 4–5 mg. of antibody-antigen precipitate. Supernatant equivalent to about 100 mg. of tumour tissue was generally used. The antibody-antigen precipitates were washed twice with 0·9% NaCl and then successively with hot and cold ethanol and dried with ether.

The tissue debris which had been sedimented by spinning for 10 min. at 12 000 *g* was precipitated with trichloroacetic acid at a final concentration of 5% (w/v). The precipitate was washed successively with boiling 5% (w/v) trichloroacetic acid, with hot and cold ethanol and dried with ether.

Preparation of microsomal particles. Mice were killed by a blow on the head and drained of blood. The tumour tissue was excised quickly, chilled in 0·39 M-sucrose, minced and pushed through stainless-steel wire netting (60 mesh). An equal weight of buffered sucrose (0·35 M-sucrose, 35 mM-$KHCO_3$, 8 mM-$MgCl_2$, 25 mM-2-amino-2-hydroxymethyl-propane-1:3-diol (tris)–HCl buffer, pH 7·8, 25 mM-KCl) was added and the cells were further dispersed by homogenizing in a Potter–Elvehjem-type homogenizer with a loosely fitting Teflon pestle at 1000 rev./min. for 20 sec. Since it is difficult to rupture the tumour cells efficiently by homogenizing they were lysed with 2·5 vol. of 0·01 M-$MgCl_2$ for 2 min. and then the sucrose concentration was adjusted to 0·3 M with fivefold-concentrated buffered sucrose medium. The suspension was spun for 15 min. at 8000 *g* to remove all cells, nuclei and mitochondria and the supernatant represented 'microsomal supernatant' used for the incubation. All manipulations were carried out in the cold or in an ice bath at −3°.

Incubation of microsomes. 'Microsomal supernatant' equivalent to 1 g. of tissue was incubated with [^{14}C]amino acids, 10 μmoles of potassium phosphoenolpyruvate (Boehringer und Söhne, Mannheim, Germany), 1 μmole of adenosine triphosphate (ATP, Sigma Chemical Co.) and 20–40 μg. of phosphopyruvate kinase (Boehringer und Söhne). Incubation for 10, 20 and 40 min. was carried out at 37° in an atmosphere of CO_2/N_2 (5:95). After incubation 10 mg. of non-radioactive amino acid was added, the microsomes were spun down in a Spinco model L ultracentrifuge for 50 min. at 100 000 *g*, the pellet was washed once with sucrose and proteins of the microsomes and the supernatant were precipitated by addition of trichloroacetic acid to a concentration of 5% (w/v) and washed and dried for counting as described by Simkin & Work (1957).

Determination of radioactivity. The finely dried protein powder was plated at infinite thickness on 0·3 cm.^{2} Perspex disks. The radioactivity was determined on an automatic recording Geiger counter with a thin end window to an accuracy of at least ±5%. Counts were converted into counts/min./cm.^{2} and corrected to infinite thickness where necessary. A ^{14}C standard 1 cm.^{2} disk (10^{-3} μc/mg.) gave 850 counts/min. at infinite thickness in this counter.

RESULTS

Globulins produced by 5563 neoplasm

Potter, Fahey & Pilgrim (1957) have shown that animals bearing the 5563 plasma-cell tumour had high levels of a γ-globulin component in the serum which on paper electrophoresis migrated in the region of fast serum γ-globulins. The concentration of the serum globulin was roughly proportional to the weight of tumour mass (Nathans *et al.* 1958) and exceeded the concentration of serum albumin when the tumour was above 2–3 g. in size.

Prolonged free electrophoresis of the tumour serum in veronal buffer (0·1 M), pH 8·6, showed an asymmetric globulin boundary which suggested heterogeneity of the myeloma globulin. The serum was analysed by starch-gel electrophoresis (Smithies, 1955) and the γ-myeloma protein separated into four to five definite bands, the concentration of the bands decreasing as the mobility of the bands increased. Fig. 1 illustrates starch-gel electrophoresis of normal mouse serum (C_3H/He strain) compared with results for serum from a mouse bearing the 5563 tumour (diluted fourfold to obtain best separation of the globulin components). It can be seen that the globulins in the tumour serum form four definite bands (and probably a fifth band present in small amounts). The γ-myeloma protein may be purified from serum by chromatography on DEAE anion-exchange cellulose (Peterson & Sober, 1956) as described in the Materials and Methods section. The elution pattern is shown in Fig. 2. The components can be partially separated by chromatography on DEAE-cellulose by using a slower gradient (B. A. Askonas & J. L. Fahey, unpublished work). The γ-myeloma globulin then spreads considerably and various fractions can be obtained which on starch gel showed different

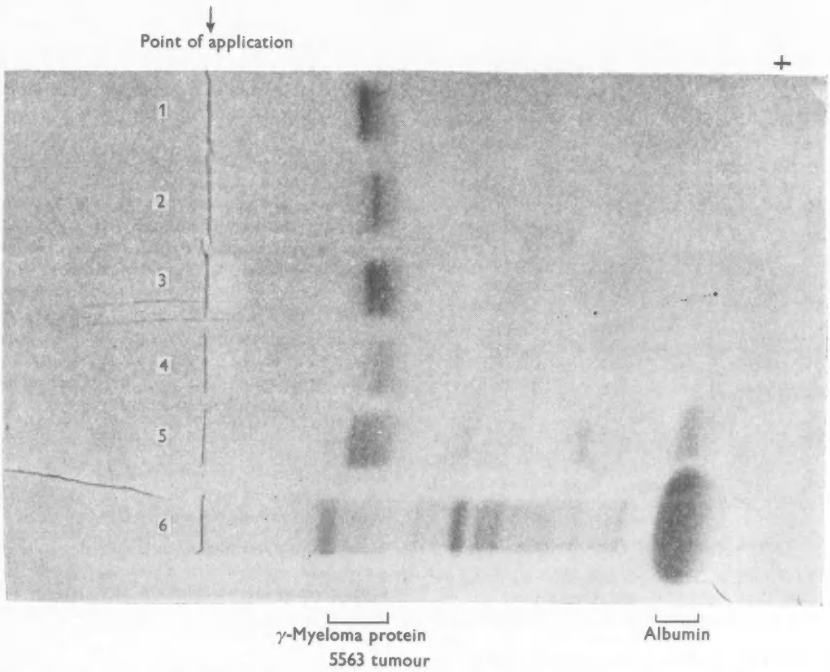

Fig. 1. Characteristics of γ-myeloma protein in serum of mice bearing 5563 plasma-cell neoplasm on starch-gel electrophoresis. 6, Normal mouse serum (C_3H/He). 5, Serum from mouse bearing 5563 plasma-cell neoplasm, diluted fourfold to obtain optimum separation of globulin molecules. 1–4, γ-Myeloma-protein fractions purified by chromatography on DEAE anion-exchange cellulose (Peterson & Sober, 1956) by a slow gradient column (B. A. Askonas & J. L. Fahey, unpublished work). Fraction 1 is eluted from the column first, fraction 4 last. Starch-gel electrophoresis was carried out by the method of Smithies (1955), with a discontinuous buffer system and 0·05 M-glycine–NaOH buffer, pH 8·9, in the gel.

mobilities (Fig. 1). It was almost impossible to obtain in this way fractions containing only one component.

The pattern on starch gel is reminiscent of the banding seen with haptoglobin polymers. The molecular weight of two extreme fractions of myeloma proteins separated by chromatography was therefore kindly determined by Dr P. Charlwood [fractions 1 and 4 (see Fig. 1)]. The two fractions had sedimentation constants of 6·5 and 6·6 respectively and thus no difference in molecular weight could be detected between the components. This S_{20} is indicative of a molecular weight of approximately 160 000 and is the same as that of normal mouse γ-globulin.

No difference in immunological reactivity could be detected between the components. The fractions were tested by immune diffusion in agar gel with antiserum against (1) pure normal mouse γ-globulin and (2) 5563 myeloma globulin, and gave reactions of identity. This suggests that the globulin components have very similar structure and antigenic groupings. At present it is not known what the difference in electric charge is due to.

The serum globulin pattern was followed over many transplant generations (15–20) throughout a period of 2 years. Fig. 3 shows a comparison of sera collected from tumour-bearing animals over a period of 2 years. It can be seen that the globulin components have the same mobility in all the sera. Since it is difficult always to get identical loading of the starch gel it is hard to judge whether minor quantitative changes have occurred or not. The chromatographic behaviour of the γ-globulin components on DEAE-cellulose has also remained unaltered over the years.

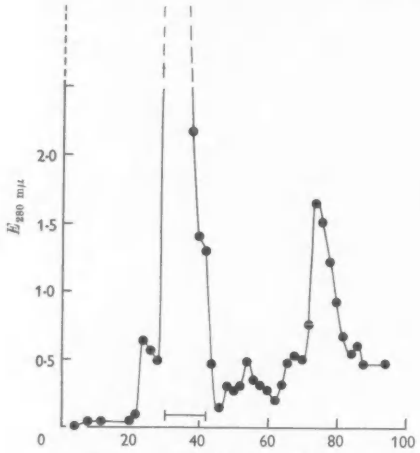

Fig. 2. Purification of γ-myeloma protein in serum of mice carrying 5563 plasma-cell neoplasm. Chromatography was carried out on a 4 g. column of DEAE ion-exchange cellulose (Peterson & Sober, 1956). Serum (3 ml.) from mice carrying the 5563 neoplasm was dialysed against the starting buffer, 0·01 M-potassium phosphate, pH 6·5. Gradient elution was carried out with 0·2 M-potassium phosphate buffer, pH 6·5, as described in the Materials and Methods section. Fractions (3 ml.) were collected and the protein elution was followed by measuring extinction of the fractions at 280 mμ. The elution pattern is presented in the diagram. Bracket (⊢⊣) indicates the fractions pooled to prepare γ-myeloma protein.

Formation of myeloma protein by plasma-cell tumour in vitro

The 5563 plasma-cell tumour was sliced and incubated in the presence of [^{14}C]amino acids as described in the Materials and Methods section. Myeloma protein was isolated by specific precipitation with antiserum. The slices actively incorporated radioactive amino acid in myeloma protein and general tissue protein insoluble in 0·9% sodium chloride (see Fig. 4 for a typical time curve). By knowing the specific radioactivity and the amount of the proteins isolated the amounts of radioactivity incorporated into tissue protein and myeloma protein may be compared. Table 1 shows several typical experiments. It can be seen that 34–52% of the total protein synthesis by the cell results in formation of the specific γ-myeloma proteins which are secreted by the cell while the cell population is dividing rapidly. Similar ratios between antibody and tissue-protein formation are found in lymph glands and spleen from hyperimmunized animals. Since these tissues contain a lot of cells forming cellular proteins other than the antibody-forming plasma cells, it suggests that the

Table 1. *Relative amounts of radioactive amino acids incorporated into general tissue protein and γ-globulin by tumour slices* in vitro

Total radioactivity represents weight of protein isolated (mg.) × specific radioactivity of protein.

In the four experiments 3–4 mg. of γ-globulin–antibody precipitate was isolated (4–6 mg. of tissue protein insoluble in 0·9% NaCl soln.).

Expt.	Radioactivity (counts/min./mg.) γ-Globulin–antibody ppt.	Tissue protein	Ratio of total radioactivity incorporated into tissue protein and γ-globulin
A	527	580	1·9
B	774	600	0·9
C	725	760	1·3
D	1530	1459	1·4

tumour cells are not quite as active in protein secretion as normal plasma cells.

An approximate figure for the amount of γ-globulin secreted by the tumour plasma cells in the system *in vitro* can be calculated. Since our counter registered 850 counts/min. for a ^{14}C standard of 10^{-3} μc/mg., the amount of added radioisotope taken up can be calculated {μc of [^{14}C]amino acid taken up = $10^{-3}/850 \times$ radioactivity of protein (counts/min./cm.^{2}) × mg. of protein isolated}. In a number of experiments with [^{14}C]glycine and algal [^{14}C]protein hydrolysate about 0·7–1 % of the

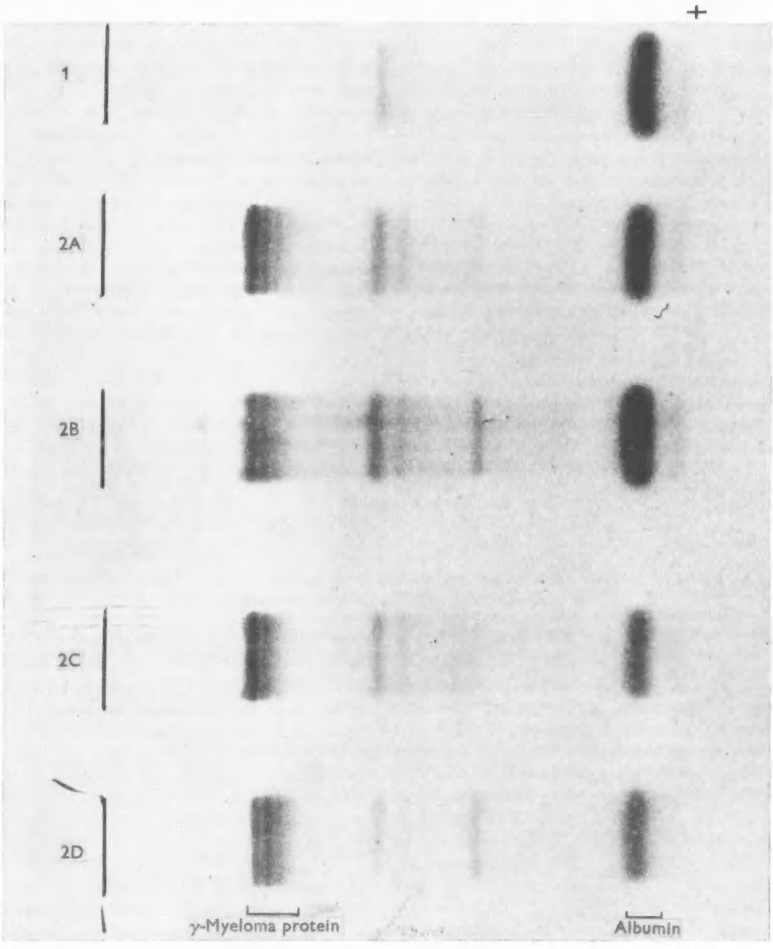

Fig. 3. Comparison by starch-gel electrophoresis of sera from 5563 tumour-bearing animals over a period of 2 years. 1, Normal mouse serum (C_3H/He), diluted fivefold. 2, Sera from mice bearing 5563 plasma-cell neoplasms (different transplantation generations). Date of killing animals: A, June 1960; B, January 1960, small tumour (1–2 g.); C, February 1959; D, August 1958. The γ-myeloma protein concentration in the sera was estimated by determining the total protein concentration with the biuret reagent and the percentage of myeloma protein by densitometry readings of stained paper-electrophoretic strips. The sera were diluted three- to seven-fold to give similar myeloma-protein concentrations for starch-gel electrophoresis. 2B, Animal had a small tumour and therefore serum was diluted only threefold; hence the normal serum components show up more strongly.

added isotope is incorporated into the γ-globulin in 5 hr. by 100 mg. of tissue. With [^{14}C]valine the values are as high as 1·7 %. This higher uptake may be explained by the fact that the 5563 myeloma protein contains twice as much valine as glycine (10·6 % of valine, 4·5 % of glycine; Nathans *et al.* 1958). In these experiments a mixture of amino acids of known composition was added to the medium so that the specific radioactivity of the [^{14}C]amino acid was known. With glycine, the medium contained 1 μc of [^{14}C]glycine and a total of 120 μg. of glycine. If we assume that the specific radioactivity of free amino acid within the cells is half that in the medium on basis of work with other tissues (Askonas & Humphrey, 1958), it may be calculated that about 1·5–2·0 mg. of myeloma protein is produced by the plasma-cell tumour *in vitro*/g. wet wt./24 hr.

Effect of nucleic acid and amino acid analogues on protein synthesis by tumour tissue

Various analogues were added to the flasks at the beginning of the incubation and incorporation of [^{14}C]amino acid into myeloma protein and tissue protein was determined at various time intervals and compared with results in control flasks to which no analogue had been added. Fig. 4 shows results of a typical time curve obtained when tumour tissue is incubated in the presence of 3·0 mM-6-azauracilriboside [I am indebted to Professor F. Šorm (Prague) for a sample of azauracilriboside]. There is very little effect for the first 2 hr.; after that the rate of synthesis of both γ-globulin and tissue protein falls off in the presence of 6-azauracilriboside; at 3 hr. there is about 30 % inhibition and at 6 hr. there is about 50 % inhibition of protein synthesis. Both the tissue-protein and myeloma-protein formation are equally affected, which suggests that protein synthesis of the cell generally is poisoned by ribonucleic acid analogue and continues only at a slow rate. 6-Azauracil was less effective (Table 2) but also caused some inhibition. Fig. 5 shows the effect of 8-azaguanine on the system. It also inhibits the formation of γ-myeloma protein and tissue protein, although it appears to take somewhat longer to have an effect. At 4 hr. there is only 15 % inhibition of protein synthesis, but thereafter the rate of synthesis falls off rapidly. Another ribonucleic acid analogue, 6-mercaptopurine, had barely any effect on protein synthesis during the time period tested (Table 2). Various amino acid analogues showed very little if any effect (Table 2). This may be due to the fact that they are incorporated into the γ-globulin. A small amount of *p*-fluoro-[^{14}C]phenylalanine could be shown to be incorporated into the proteins by the plasma-cell tumour. Incubation of tumour slices was carried out in the usual way, except that the medium contained a synthetic L-amino acid mixture free of phenylalanine. *p*-Fluoro-[^{14}C]-phenylalanine (1·3 μc) was added to the incubation

Table 2. *Effect of various nucleic acid and amino acid analogues on formation of myeloma protein and general tissue protein by tumour slices* in vitro

Control flasks at each time point were incubated in the usual medium (see Materials and Methods section). The analogues tested and [^{14}C]amino acids were added to the flasks at the beginning of the incubation period (at 0 hr.). N.D., Not done.

Addition	Concn. (mM)	Incubation time (hr.)	Inhibition of incorporation of [^{14}C]amino acid into protein (%): Myeloma protein–antibody ppt.	Inhibition of incorporation of [^{14}C]amino acid into protein (%): Tissue protein
Trifluoroleucine	4·5	7·5	4	5
p-Fluorophenylalanine	2·7	6	13	N.D.
		8	20	N.D.
Thienylalanine	3	6	10	N.D.
6-Azauracilriboside	3·3	3	32	29
	3·3	6	50	50
	3	1	0	0
	3	2·5	15	0
	3	4	30	20
6-Azauracil	3	1	0	0
	3	2·5	17	0
	3	4	13	15
8-Azaguanine	3	4	15	15
	3	6·25	40	34
6-Mercaptopurine	3	6	10	N.D.
Bromouracil	3	6	10	10

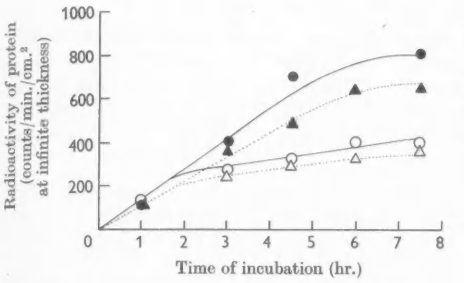

Fig. 4

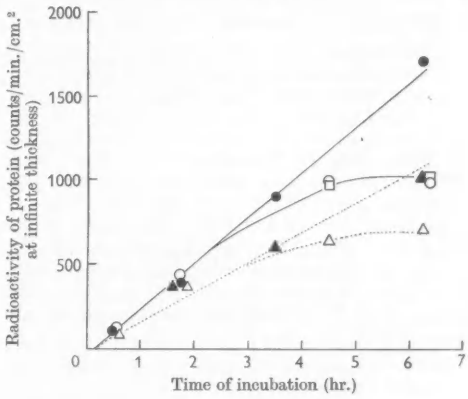

Fig. 5

Fig. 4. Effect of 6-azauracilriboside on protein synthesis by tumour slices *in vitro*. ●, Radioactivity of γ-myeloma protein–antibody ppt. ○, Radioactivity of myeloma protein after addition of 3 mM-6-azauracilriboside to incubation mixture at zero time. ▲, Radioactivity of tissue protein insoluble in 0·9% NaCl soln. △, Tissue protein after addition of 3 mM-6-azauracilriboside at zero time. Sliced tumour tissue (100 mg.) was incubated in the presence of 1 μc of [^{14}C]glycine. Myeloma protein (3·5 mg.) and tissue protein (4 mg.) were isolated as described in the Materials and Methods section.

Fig. 5. Effect of 8-azaguanine on protein synthesis by tumour slices *in vitro*. ●, Radioactivity of γ-myeloma protein–antibody ppt. ○, Radioactivity of myeloma protein if incubation was carried out in the presence of 3 mM-8-azaguanine. ▲, Radioactivity of tissue protein insoluble in 0·9% NaCl soln. △, Tissue protein, when incubated in the presence of 3 mM-8-azaguanine. □, Myeloma protein; incubation was carried out in the presence of 3 mM-8-azaguanine, but 10 mM-guanine was added after incubation for 3 hr. Conditions of incubation and isolation of proteins as described in the Materials and Methods section. Sliced tumour (110 mg.) was incubated in the presence of 1 μc of [^{14}C]valine.

flasks and the radioactivity of the γ-globulin–antibody precipitate was determined. A heterologous antibody–ovalbumin precipitate was first made to obtain values for non-specific coprecipitation. The heterologous precipitate had only 4–5 counts/min. After incubation for 6 hr. both the tissue protein (4 mg.) and the γ-globulin precipitate (4 mg.) had about 70 counts/min./mg. This is equivalent to about 0·04% uptake of *p*-fluoro-L-[^{14}C]phenylalanine in 6 hr. by 100 mg. of tumour tissue into both γ-globulin and tissue protein.

Studies with microsomal fraction of tumour tissue

It is of considerable theoretical importance to find out which intracellular sites form γ-globulin and antibody and to determine its characteristics. It was attempted to study this problem with subcellular preparations of tumour tissue. Tumour tissue (5563) was chosen for this since it provides a fairly homogeneous population of plasma cells whereas tissues which usually form γ-globulin and antibody are composed of many cell types.

The microsomal particles were fairly active in incorporating [^{14}C]valine or [^{14}C]leucine or both into trichloroacetic acid-precipitable protein (Table 3), the system described by Zamecnik & Keller (1954) being used as energy source. There was no incorporation in the absence of ATP and phosphoenolpyruvate, and phosphoenolpyruvatekinase was necessary for optimum incorporation. Incorporation did not continue beyond 15–20 min.; at this time 0·1% of the added isotope had been incorporated. This compares favourably with the liver system, but the microsomal particles appear to be more unstable than these obtained from liver by homogenization. It was not possible to spin down the microsomal pellet at 100 000 *g* and then recombine it with supernatant as described for liver by Campbell, Greengard & Kernot (1960). After ultracentrifuging, the microsomal pellet from the 5563 neoplasm did not incorporate any radioactivity into its proteins.

It was not possible to demonstrate formation of γ-globulin by the subcellular particles. After incubation, the microsomal pellet was extracted with 0·1 M-sodium carbonate buffer, pH 9·5, treated with ultrasonics and incubated for a few minutes with 100 μg. of ribonuclease. Carrier myeloma

Table 3. *Incorporation of* [^{14}C]*amino acids into protein by the microsomal fraction of* 5563 *neoplasm*

Microsomal supernatant was prepared as described in the Materials and Methods section. Each incubation flask contained microsomal supernatant (2·6 ml.) derived from 0·6 g. of tumour tissue (5563), and 1 μc of L-[^{14}C]valine and L-[^{14}C]leucine. The yield of washed and dried protein from the microsomal pellet and the supernatant was 6 and 15 mg. respectively. In 20 min. 0·1 % of the isotope was taken up into the microsomal protein.

Tube no.	Addition	Incubation time (min.)	Radioactivity of protein (counts/min./mg.)	
			Microsomal	Supernatant
1	—	20	22	35
2	10 μmoles of phosphoenolpyruvate, 1 μmole of ATP, 20 μg. of phosphoenolpyruvatekinase	20	376	35
3	10 μmoles of phosphoenolpyruvate, 1 μmole of ATP, 20 μg. of phosphoenolpyruvatekinase	35	386	35

Table 4. *Relative incorporation of* [^{14}C]*amino acids into various protein fractions of microsomes*

Incubation: Microsomal supernatant (from 0·8 g. of 5563 neoplasm) was incubated for 20 min. at 37° in the presence of 1·5 μc of L-[^{14}C]valine. Control flasks had no further additions. Flasks with full medium also contained 10 μmoles of phosphoenolpyruvate, 2 μmoles of ATP and 30 μg. of phosphoenolpyruvatekinase. The microsome pellet was spun down at 100 000 *g* for 60 min. after incubation.

Microsome extract: Carrier 5563-myeloma protein (500 μg.) was added for eventual precipitation with antiserum to a pool of four incubation flasks. The microsomal pellet was treated with 0·1 M-sodium carbonate buffer, pH 9·5, and ultrasonic vibration (25 kcyc., 500 w) and 100 μg. of ribonuclease. Before precipitation of the γ-globulin with antiserum, nucleoprotein was removed by precipitation at pH 5·6 to avoid its coprecipitation.

	Radioactivity of protein (counts/min./mg.)	
	Control	Full medium
Total microsomal protein (trichloroacetic acid ppt.)	48	210
Microsome extract		
Ovalbumin–antibody ppt.	0	1·2
γ-Globulin–antibody ppt.	0	2·6
0·1 M-Sodium pyrophosphate, pH 8·3	4	52
Residual protein (trichloroacetic acid ppt.)	38	227

protein was then added (500 μg.) and the pH was adjusted to pH 5·6 to remove by precipitation the bulk of the nucleoprotein, which tends to coprecipitate in antibody–antigen reactions. This procedure removes only about 5 % of the γ-myeloma protein, when tested in the presence of ^{131}I-labelled myeloma protein. After spinning for 10 min. at 10 000*g* the supernatant is clear. A heterologous antibody precipitate was first made as a control (rabbit antiovalbumin and ovalbumin were used). The mouse myeloma protein was then precipitated with specific rabbit antiserum, but no radioactivity was associated with this precipitate. A small amount of radioactivity could be extracted from the pellet with 0·1 M-pyrophosphate buffer, pH 8·3, but most of the radioactivity was associated with protein insoluble in 0·9 % sodium chloride and particles (Table 4). The failure to demonstrate γ-globulin synthesis by the microsome may be due to the instability of the particles prepared from the tumour tissue, so that only some stages of protein synthesis are being carried out, and it should not be concluded from such experiments that γ-globulin is not formed in the microsomal particles. It is hoped that more stable preparations will be achieved eventually.

'*Bound*' 5563 *myeloma protein in the microsomal fraction*

A small amount of myeloma protein is released when the microsomal fraction is treated with sodium carbonate buffer, pH 9·5, ultrasonics and ribonuclease or with 0·5 % deoxycholate. The microsomal fraction from the tumour looks very similar to that of liver in electron micrographs. It consists of ribosome particles and some membranous vesicles which probably contain the protein to be secreted by the cell. Many substances such as alkali, tris, ethylenediaminetetra-acetic acid, deoxycholate, which break the structure of membranes and release nucleic acid from ribosomes, may release the myeloma protein and it is therefore not possible to say whether it is truly 'bound' by any linkage or simply enveloped by the membranous vesicles. On starch-gel electrophoresis the bound myeloma protein gives the same pattern as serum myeloma protein, which suggests that the different components are formed as such by the cell and that the heterogeneity is not due to enzymic action or formation of complexes in the extracellular fluid or in the circulation.

The amount of 'bound' myeloma protein in the microsomal fraction could be determined by titration with specific rabbit antiserum and was found

to be 80 μg. of myeloma protein/g. wet wt. of tumour tissue, representing 1 % of the total microsomal protein. Similar values were found for albumin released from microsomal fractions of normal liver by ultrasonics (Campbell *et al.* 1960). By deoxycholate treatment Peters (1957) obtained 170 μg. of albumin from chick-liver microsomes. About 50 times this amount of myeloma protein is found free in the cytoplasm and extracellular fluid per g. wet wt. of tumour tissue.

DISCUSSION

The globulins secreted by the 5563 plasma-cell neoplasm were found to be heterogeneous and to consist of five definite protein bands on starch-gel electrophoresis. In human myeloma it has generally been thought that myeloma proteins migrating as γ-globulin molecules are homogeneous. Most myeloma sera have, however, been analysed by paper electrophoresis or free electrophoresis, where such small molecular differences would not be detected. One human myeloma protein migrating in the γ-globulin region has been reported to be heterogeneous on starch-gel electrophoresis, giving a pattern very similar to the 5563 protein (Owen, Got & Silberman, 1958). It is not clear what causes the difference in electric charge of the 5563 components. The fractions were found to have the same molecular weight as normal mouse γ-globulin (S_{20} 6·5) and no antigenic differences were detected by immunological techniques. It is possible that there are minor differences in amino acid sequence or in non-peptide molecules forming complexes with the protein. Enzymic degradation of the components as described by Porter (1959) for rabbit antibody is being studied to investigate any structural differences.

The observation that the physicochemical properties of the globulin molecules secreted by the 5563 plasma-cell tumour remain constant over many transplant generations throughout a period of 2 years is of special interest. The other lines of plasma-cell tumours investigated over many transplant generations also are associated with constant and characteristic serum-protein patterns on electrophoresis even when transplanted into hybrid hosts (Potter & Fahey, 1960). One is dealing with lines of differentiated plasma cells which divide rapidly. Various possibilities have been put forward to explain the wide heterogeneity of physical and biological properties of γ-globulin and antibody in normal animals. It has been suggested, for example, that γ-globulin molecules can take on numerous different configurations or that γ-globulin-forming cells are hypermutable, resulting in variability of globulin molecules. The findings with the transplantable plasma-cell neoplasms indicate that the differentiated plasma cell is stable in its genotype and produces a constant and stable pattern of globulin molecules with no evidence for unstable molecular configurations.

The experiments *in vitro* have shown that the γ-globulin is produced by the tumour tissue and is not formed as a reaction of the host against the tumour graft. Although the plasma-cell tumour is growing rapidly it is simultaneously secreting considerable amounts of globulins into the serum so that the serum-globulin concentration is higher than the albumin concentration when the tumour weight is above 2–3 g. Tissue slices of the 5563 neoplasm incubated *in vitro* in the presence of [^{14}C]amino acid formed 1·5–2·0 mg. of γ-globulin protein/g. of tissue/24 hr.; 34–52 % of the total protein synthesized by the plasma-cell tumour is represented by the γ-myeloma protein secreted into the serum (see Table 1).

The γ-globulin production is slightly lower than albumin formation by normal liver in similar experimental systems. Peters (1957) and Campbell & Stone (1957) reported that liver slices (from chicken and rat) form 3–5 mg. of albumin/24 hr./g. wet wt. of tissue. The plasma-cell tumour shows similar protein synthetic activity to liver hepatoma, which was less active in albumin synthesis (20–50 %) than is normal liver (Campbell & Stone, 1957).

Since nucleic acid and amino acid analogues are being used frequently in cancer therapy in cases of myeloma and leukaemia, it was of interest to study the effect of these analogues on protein synthesis by the tumour tissue *in vitro*. This provides an easy method for testing the toxicity of these substances for tumour tissue, whereas in tests *in vivo* inhibition of tumour growth or tumour regression has to be investigated on a large number of animals.

Table 2 and Figs. 4 and 5 have shown that the ribonucleic acid analogues 8-azaguanine- and 6-azauracil-riboside inhibited both globulin formation and general protein synthesis after the first 2–3 hr. of incubation. Similar inhibition was observed when tests for incorporation of [^{14}C]valine, [^{14}C]glycine or algal [^{14}C]protein hydrolysate were made.

The mammalian system differs from the bacterial system, in which Richmond (1959) was able to show inhibition of formation of lytic enzymes in *Bacillus subtilis* within 5 min. of the addition of a low concentration of 8-azaguanine, before incorporation of the analogue into ribonucleic acid could be demonstrated. It is possible that in the slower growing tumour there are bigger pools of nucleic acid precursors than in bacteria so that protein synthesis can proceed for some time. In mammalian systems far higher (10–20-fold) con-

centrations of azaguanine than in the bacterial system have to be used to give any inhibition. As in the bacterial system, azaguanine leads to poisoning of general protein synthesis by the cell.

6-Azauracilriboside was found to have anti-tumour activity in mice with ascites tumours, whereas 6-azauracil was less effective (Šorm & Keilova, 1958). This was similar to results *in vitro* in this study presumably because the azauracil has to be converted into the riboside before utilization. It has been shown by Schindler & Welch (1957) that only the riboside inhibits growth of sarcoma 180 cells in tissue culture, and by Brockman, Sparks & Simpson (1957) that mouse leukaemic cells resistant to 8-azaguanine were not able to convert it into the riboside, whereas sensitive strains formed 8-azaguanineriboside. It is possible that the 6-mercaptopurine had no effect on protein synthesis by the 5563 tumour because the tumour was unable to form the riboside. The riboside was not available for testing.

Dutton, Dutton & George (1958) have reported inhibition (35–50 %) of antibody formation by spleen cells from immunized animals with 8-azaguanine and *p*-fluorophenylalanine (about 3 mM) after incubation for 24 hr. Hence the 5563 neoplasm appears to have similar sensitivity to azaguanine, as do normal globulin-forming cells, but no effect on protein synthesis was detected by any of the amino acid analogues tested on the 5563 tumour slices during incubation for 6 hr. (Table 2). It could be demonstrated that small amounts of *p*-fluoro-[^{14}C]phenylalanine are incorporated into the γ-myeloma protein by the 5563 plasma-cell tumour, and hence γ-globulin continued to be formed in the presence of *p*-fluorophenylalanine. Vaughan & Steinberg (1960) have had similar results and have been able to isolate peptides containing tritiated *p*-fluorophenylalanine from ovalbumin and lysozyme formed by hen's oviduct.

SUMMARY

1. The 5563 plasma-cell neoplasm, transplantable in mice, has been shown to form heterogeneous globulins separating into five definite bands on starch-gel electrophoresis. The different components can be partly separated by chromatography on anion-exchange cellulose and were found to have the same sedimentation constant (S_{20} 6·5) and the same immunological groupings.

2. The protein production of this plasma-cell tumour could be followed over many transplant generations (15–20). Globulin components with the same mobilities were produced by the neoplastic tissue of tumour-bearing mice over a period of two years. This suggested that the genotypes of differentiated plasma cells is stable, with no evidence for mutation in protein synthetic capacity although there is rapid cell division.

3. By incubating slices of tumour tissue and isolating the myeloma protein by specific precipitation with antisera, it could be shown that the plasma cells form the myeloma protein at a considerable rate (1·5–2·0 mg./g. wet wt. of tissue/day).

4. Amino acid analogues had no significant effect on protein synthesis by the plasma cells during incubation for 6–7 hr. *in vitro*. *p*-Fluoro-[^{14}C]phenylalanine was incorporated into the myeloma globulin at a very low rate. Ribonucleic acid analogues (3 mM-azauracilriboside and -azaguanineriboside) had no effect for the first 2–3 hr. but after that inhibited both the formation of tissue protein and myeloma protein to a similar extent, causing a 40–50 % inhibition of protein synthesis after 6 hr. The tumour tissue appears to behave similarly to bacterial systems and normal tissue.

5. A microsomal fraction could be prepared by lysis of cells with dilute magnesium chloride that incorporated [^{14}C]amino acids into protein insoluble in 0·9 % sodium chloride, but no incorporation into the globulin could be demonstrated. The microsomal fraction contained 'bound' myeloma protein released by extraction with alkali and ultrasonic vibration.

I am greatly indebted to Dr M. Potter for sending us tumour-bearing animals and breeding pairs of his mouse strains and thus making this study possible. I should like to thank Mrs Marion Perryman for her excellent assistance with all the experiments and Dr P. A. Charlwood for doing the ultracentrifugal analyses of the 5563 protein fractions.

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Comparison of the Aerobic and Anaerobic Reduction of Oxidized Glutathione in Liver Homogenates

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Since the glutathione of animal tissues, other than blood, is predominantly in the reduced form (Bhattacharya, Robson & Stewart, 1955; Martin & McIlwain, 1959), the rate of reduction of oxidized glutathione in respiring tissues must be rapid enough to counteract the enzymic (Ames & Elvehjem, 1945, 1946) and non-enzymic (Barron, 1951) oxidation of reduced glutathione. In most studies of reduction of oxidized glutathione, non-respiring glutathione-reductase preparation (Rall & Lehninger, 1952; Van Heyningen & Pirie, 1953; McIlwain & Tresize, 1957) or anaerobic conditions (cf. Vennesland & Conn, 1954) have been employed. Hitherto few investigators have compared the aerobic and anaerobic reduction of oxidized glutathione in tissue preparations capable of respiration. Martin & McIlwain (1959) found little difference in the aerobic and anaerobic rates of reduction of endogenous oxidized glutathione in slices of guinea-pig cerebral cortex. In avocade mitochondria supplemented with citrate and triphosphopyridine nucleotide (cf. Vennesland & Conn, 1954) the aerobic and anaerobic rates of reduction of oxidized glutathione were approximately equal. In the present investigation the aerobic and anaerobic rates of reduction of added oxidized glutathione in liver homogenates have been compared under a variety of conditions.

MATERIALS AND METHODS

Glutathione. Reduced glutathione (GSH) was obtained from The Distillers Co. Ltd. Oxidized glutathione (GSSG) was prepared from GSH by a modification of the method of Pirie (1931) without added Cu^{2+} ions. To a solution of GSH (2%, w/v) was added an equivalent amount of 3% (w/v) H_2O_2, and the solution kept in a refrigerator at 2° for 12 hr. Oxidation at this stage was incomplete and the residual SH-groups were estimated potentiometrically. More H_2O_2 was added, a little less than was necessary to complete the reaction, and the solution was stored for a further 12 hr. at 2°. The purpose of this procedure was to avoid side reactions. The final product always contained a small percentage of GSH. It was considered preferable to use a solution of GSSG containing a small percentage of SH-groups rather than one containing side products of oxidation. With this method of preparation the total (GSSG + GSH) present in the final solution was equivalent to not less than 95% of the original GSH.

Sodium glucose 6-phosphate. This was prepared from the barium salt (British Drug Houses Ltd.) by ion exchange with Dowex 50.

Suspension media. 'Phosphate saline' contained 100 vol. of 0·155M-KCl, 10 vol. of 0·1M-potassium phosphate buffer, pH 7·4, and 1 vol. of 0·1M-$MgCl_2$. 'Bicarbonate saline' was a modification of the saline of Krebs & Henseleit (1932). The stock solution consisted of 100 ml. of 18% (w/v) KCl, 80 ml. of 1·15% (w/v) KCl, 15·4 ml. of 0·2M-KH_2PO_4, 20 ml. of 3·82% (w/v) $MgSO_4,7H_2O$, 60 ml. of 1·3% (w/v) $NaHCO_3$ and 1960 ml. of water. Before use 16 vol. of 1·3% (w/v) $NaHCO_3$ were added to 100 vol. of the stock solution, and the mixture was gassed with N_2+CO_2 (95:5) at 0° for 15 min. Phosphate saline was used in all experiments unless stated otherwise.

Preparation of homogenates. Immediately after removal from the animal the liver was cooled in 0·9% KCl at 0°, dried with filter paper and minced in a chilled Fischer mincer (Jouan, Paris). The minced tissue was weighed and homogenized with 8–30 vol. of ice-cold suspension medium in a chilled stainless Potter–Elvehjem homogenizer.

Incubations. These were carried out in Warburg apparatus at 30°. Vessels containing phosphate saline were gassed with O_2 or N_2 and those containing bicarbonate saline were gassed with O_2+CO_2 (95:5) or N_2+CO_2 (95:5).

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A stick of yellow phosphorus was placed in the centre well of vessels gassed with N_2 or N_2+CO_2. The centre wells of vessels gassed with O_2 contained 0·2 ml. of 2 N-NaOH and filter paper. The incubation mixture consisted of 3·0 ml. of homogenate, and substrates and saline to make the final volume up to 4·0 ml. The concentrations of GSSG and GSH solutions added were usually 0·05–0·10 M and the oxidized triphosphopyridine nucleotide (TPN^+) solution was 1·5 mM. In most experiments other substrates were added as 0·1–0·2 ml. of a 0·1–0·2 M-solution. Only GSH and GSSG were added to the side arm. The vessels were kept at 0° after the addition of the homogenate, before gassing and incubation. The contents of the side arm were added immediately before incubation. At the end of the incubation the vessels were cooled in ice, and the reaction was stopped by adding 1·0 ml. of 10% (w/v) metaphosphoric acid. Control to estimate endogenous GSH consisted of a mixture of 1 ml. of 10% (w/v) metaphosphoric acid, 1 ml. of saline and 3 ml. of homogenate. The mixture to estimate initial GSH consisted of 1 ml. of 10% (w/v) metaphosphoric acid, 3 ml. of homogenate, GSH solution and saline to make the final volume up to 5 ml. The vessels with both controls were kept at 0°. Estimations of GSH were done at the same time for both incubated and unincubated mixtures. We considered it better to estimate initial GSH rather than determine its value by summing up endogenous and added GSH.

Estimation of reduced glutathione. The precipitated proteins were removed by centrifuging at 20 000*g* at 2° for 15 min. in the refrigerated International centrifuge. A portion (0·25–0·5 ml.) of the supernatant was diluted to 4·3 ml. with water (final pH 1·5) and titrated potentiometrically against 0·1 M-$HgCl_2$ by a modification of the method of Cecil (1955). The apparatus used was the same as that described by Cecil (1950). The method described by Cecil (1955) for cysteine did not give sharp end points with GSH but these were obtained with the following modifications.

The gold wire of the electrode was fused at one end to form a ball about 0·6 mm. in diameter. The electrode was prepared by heating the gold (cf. Cecil, 1955) and dipping it for 40 sec. into mercury. Although end points were sharp with aqueous GSH solutions, unsatisfactory end points were obtained with deproteinized solutions. This was apparently due to failure of the mercury–thiol electrode to form spontaneously in deproteinized solutions (cf. Cecil, 1955). The difficulty was overcome by prior immersion of the electrodes in aqueous GSH (approx. 0·2 mM). This ensured that there was a layer of $(GS)_2Hg$ on the surface of the electrode before the titration was started. The immersion was continued until a steady potential had been reached (approx. 100 mv versus the control reference electrode), after which a little $HgCl_2$, equivalent to about 15% of the GSH present, was collected. Electrodes prepared in this way could be used for about 20 titrations provided that the concentration of free Hg^{2+} ions was kept low. After prolonged use of the electrode the initial potential rose and the end point became less sharp.

If the titration is continued beyond the equivalence point, corresponding to $(GS)_2Hg$, a second equivalence point is reached, corresponding to $(GS)_2Hg_2$. The first end point was always used as a more reliable measure of GSH (cf. Stricks & Kolthoff, 1953). Care must be taken in titrating small amounts of GSH because the inflexion between the two end points is small.

The error of the method was about 1% with 1·0 μmole and 5% with 0·2 μmole of GSH. Recoveries of added GSH from manometer-flask contents containing pigeon-liver homogenate (approx. 75 mg. dry wt./flask) were 90, 98 and 100% with concentrations of GSH of 0·5, 2·5 and 5 mM respectively.

Estimation of oxidized glutathione. This was carried out in aqueous solution according to Cecil (1955).

RESULTS

Aerobic disappearance of added reduced glutathione in fresh and boiled liver homogenates

GSH is oxidized non-enzymically by molecular oxygen (Barron, 1951) and by certain substances which may be present in biological systems. The oxidation of GSH is also accelerated by certain tissue preparations (Ames & Elvehjem, 1945, 1946).

Therefore before measuring the reduction of GSSG in liver homogenates the fate of added GSH was investigated (Table 1). There was no disappearance of GSH incubated aerobically with fresh pigeon-liver homogenate, but with boiled pigeon-liver homogenate 15–17% of the initial GSH disappeared after 60 min. The percentage of GSH disappearing on incubation with the boiled homogenate was independent of the concentration of tissue or GSH. These findings suggest that there is a mechanism in fresh pigeon-liver homogenate for the reduction of GSSG which is more rapid than the non-enzymic oxidation of GSH.

On incubation with fresh rat-liver homogenate (Table 1) 36% of the initial GSH disappeared in 60 min., whereas with boiled rat-liver homogenate only 18% of the initial GSH disappeared in 60 min. Thus, in contrast with pigeon-liver homogenate, the disappearance of GSH is twice as rapid in fresh as in boiled rat-liver homogenate. It is probable that the rapid removal of GSH is a property of rat-liver homogenate and not of the intact tissue since Bhattacharya *et al.* (1955) found that glutathione in rat liver is predominantly in the reduced form. This idea is supported by the observation (Expt. 3, Table 1) that the rate of disappearance of GSH increased with incubation time. The rate of removal of GSH between 0 and 60 min. was almost twice as rapid as the rate in the first 20 min. It seems therefore that the capacity of rat liver to oxidize GSH appears on homogenization and increases during incubation.

Comparison of the aerobic and anaerobic reduction of oxidized glutathione in homogenates of pigeon, rat and guinea-pig liver

The anaerobic reduction of GSSG is approximately linear for 20 min. in homogenates of rat, pigeon and guinea-pig liver (Table 2) but falls off after longer incubation in homogenates of both rat

Table 1. *Aerobic removal of reduced glutathione in fresh and boiled liver homogenates*

Vessels contained GSH as indicated below, 3·0 ml. of homogenate, and phosphate saline to a total volume of 4·0 ml. 'Boiled' homogenate was heated for 15 min. at 100°.

Animal	Dry wt. of tissue (mg.)	Homogenate	GSH added (μmoles)	Endogenous GSH (μmoles)	Initial GSH (μmoles)	Incubation time (min.)	Final GSH (μmoles)	GSH removed (μmoles)	GSH removed (%)
Pigeon	96·0	Fresh	6·0	1·8	7·8	10	7·8	0	0
						20	7·8	0	0
						40	7·8	0	0
						80	7·8	0	0
		Boiled	6·0	1·8	7·8	80	6·3	1·5	19
Pigeon	38·5	Fresh	20·0	0·7	21·0	60	20·9	0·1	0
		Boiled	20·0	0·6	20·4	10	19·5	0·9	4
						25	18·7	1·7	8
						60	17·3	3·1	15
			5·0	—	5·8	10	5·4	0·4	7
						25	5·1	0·7	12
						60	4·8	1·0	17
Rat	140·0	Fresh	7·0	2·0	9·2	10	9·0	0·2	2
						20	8·6	0·6	7
						60	6·0	3·2	36
		Boiled	7·0	1·8	9·1	60	7·5	1·6	17

Table 2. *Time course of the aerobic and anaerobic reduction of oxidized glutathione in homogenates of pigeon, rat and guinea-pig liver*

Vessels contained 0·2 ml. of GSSG (approx. 0·05 or 0·025 M, containing GSSG + GSH as indicated below), 3·0 ml. of homogenate, and phosphate saline to a total vol. of 4·0 ml.

Expt. no.	Animal	Dry wt. of tissue (mg.)	GSSG added (μmoles)	GSH added (μmoles)	Endogenous GSH (μmoles)	Initial GSH (μmoles)	Incubation time (min.)	Final GSH (μmoles) N_2	Final GSH (μmoles) O_2	GSH formed (μmoles) N_2	GSH formed (μmoles) O_2
1	Pigeon	50·5	9·0	0·4	0·8	1·2	10	1·9	2·0	0·7	0·8
							25	3·2	3·2	2·0	2·0
							70	5·2	4·5	4·0	3·3
2	Pigeon	39·0	9·0	0·4	0·6	1·1	10	1·7	1·6	0·6	0·5
							25	2·1	2·0	1·0	0·9
							60	2·4	1·9	1·3	0·8
3	Rat	140·0	3·9	2·2	2·0	4·4	10	5·2	4·9	0·8	0·5
							27	6·8	3·8	2·4	−0·6
							55	8·8	1·4	4·4	−3·0
4	Rat	145·2	3·9	2·2	1·8	4·0	10	5·0	4·5	1·0	0·5
							20	5·9	3·5	1·9	−0·5
							60	7·2	1·0	3·2	−3·0
5	Rat	71·5	3·6	1·6	0·7	3·1	10	3·3	—	0·2	—
							20	3·6	—	0·5	—
							40	4·2	—	1·1	—
		133·3	—	—	1·3	3·9	10	5·1	—	1·2	—
							20	6·6	—	2·7	—
							40	8·5	—	4·6	—
6	Guinea pig	80·8	3·6	1·8	1·5	3·6	10	4·2	4·1	0·6	0·5
							20	4·8	3·8	1·2	0·2
		150·5	—	—	2·9	4·8	10	6·2	6·2	1·4	1·4
							20	7·3	6·2	2·5	1·4

and pigeon liver. Homogenate equivalent to about 50, 150 and 100 mg. dry wt. of tissue/flask was required respectively for pigeon, rat and guinea-pig liver, in order to obtain approximately equal anaerobic rates of reduction of GSSG (about 1·0 μmole of GSH/10 min.). It was difficult to assess absolutely the relative rates of reduction of GSSG in homogenates of the three tissues as this was not proportional to tissue concentration. In preliminary experiments the rates of reduction of GSSG in homogenates of pigeon, rat and guinea-pig liver prepared in 8 vol. of phosphate saline were respectively about four, six and three times the rates in homogenates prepared in 16 vol. of the saline.

Aerobically the pattern of reduction of GSSG differed widely in homogenates of pigeon, rat and guinea-pig liver (Table 2). In pigeon-liver homogenate the aerobic rate of GSSG reduction from 0 to 25 min. was about the same as (Tables 2 and 4) or slightly less than (Table 3) the anaerobic rate. With incubation periods greater than 20 min. the aerobic rate of reduction of GSSG was invariably less than the anaerobic rate (Tables 2 and 4). In rat-liver homogenate (Table 2) a small aerobic reduction of GSSG (about half of the anaerobic rate) was observed in the first 10 min. but with longer periods of incubation the removal of GSH was so rapid that even the endogenous GSH disappeared (cf. Hopkins & Elliot, 1931). In guinea-pig-liver homogenate (Expt. 6, Table 2) the aerobic rate of GSSG reduction was the same as the anaerobic rate up to 10 min. but with longer incubation GSH ceased to accumulate (150 mg. dry wt. of tissue/flask) or disappeared (80 mg. dry wt. of tissue/flask).

In all experiments therefore with all three tissues the anaerobic rate of reduction of GSSG is greater than or equal to the aerobic rate. The difference between the aerobic and anaerobic rate of reduction of GSSG is much smaller in pigeon-liver homogenate than in rat-liver or guinea-pig-liver homogenate in which GSH disappears on prolonged aerobic incubation.

Since GSH is stable under aerobic conditions in pigeon-liver homogenate (Table 1) this tissue was

Table 3. *Effect of concentration of oxidized glutathione on its aerobic and anaerobic reduction in pigeon-liver homogenates*

Vessels contained 0·1–0·4 ml. of approx. 0·05 M-GSSG (containing GSSG + GSH as indicated below), 3·0 ml. of homogenate, and phosphate saline to a total vol. of 4·0 ml.

Dry wt. of tissue (mg.)	Gas	GSSG added (μmoles)	GSH added (μmoles)	Endogenous GSH (μmole)	Initial GSH (μmoles)	Incubation time (min.)	Final GSH (μmoles)	GSH formed (μmoles)
35·7	N_2	3·6	2·1	0·7	2·8	10	3·6	0·8
						20	4·3	1·5
						50	6·3	3·5
		7·2	4·2	0·7	4·9	10	5·6	0·7
						20	6·2	1·3
						50	8·4	3·5
		14·4	8·4	0·7	9·0	10	10·0	1·0
						20	10·5	1·5
						50	12·8	3·8
40·8	N_2	3·6	1·8	0·7	2·5	12	3·7	1·2
						20	4·2	1·7
						40	4·9	2·4
	O_2	3·6	1·8	0·7	2·5	12	3·7	1·2
						20	3·9	1·4
						40	3·9	1·4
		7·2	3·6	0·7	4·1	12	5·0	0·9
						20	5·2	1·1
						40	5·1	1·0
41·5	N_2	7·2	3·6	0·8	4·5	5	5·3	0·8
						10	5·9	1·4
						20	7·3	2·8
	O_2	7·2	3·6	0·8	4·5	5	5·3	0·8
						10	6·2	1·7
						20	6·4	1·9
		14·4	7·2	0·8	7·8	5	8·6	0·8
						10	9·3	1·5
						20	9·1	1·3

used in subsequent experiments. The standard incubation period was usually 10 min. (Tables 5–7) because the rate of reduction of GSSG was approximately linear during this incubation period (Tables 2 and 3).

Effect of concentration of oxidized glutathione on its aerobic and anaerobic rate of reduction in pigeon-liver homogenates

The anaerobic rate of reduction of GSSG was not affected by increasing the amount of GSSG added per flask from 3·6 to 14·4 μmoles (Table 3). Aerobically the rate of reduction of GSSG was decreased by raising the concentration of GSSG. The reason for this effect is not clear. Perhaps the GSSG solution contained some impurity which under the action of oxygen forms an inhibitor of GSSG reduction. Alternatively the inhibition of GSSG reduction with increasing GSSG concentration may be associated with mitochondrial swelling produced by the GSH in the preparation (Lehninger & Schneider, 1959). Since increasing the GSSG concentration did not increase the rate of GSSG reduction, it is to be concluded that under the conditions summarized in Table 3 the concentration of GSSG is not rate-limiting. This is consistent with the finding of McIlwain & Tresize (1957) that a GSSG concentration of 0·2 mM is required for maximum glutathione-reductase activity in cerebral extracts. The lowest GSSG concentration used in the present experiments was 0·9 mM.

Effect of oxidized triphosphopyridine nucleotide on the reduction of oxidized glutathione in pigeon-liver homogenates

Addition of 0·0375 μmole of TPN^+/ml. (Table 4) increased both the aerobic and anaerobic rate of reduction of GSSG two- to three-fold. In the presence of added TPN^+ the aerobic rate of GSSG reduction was the same as the anaerobic rate for incubation periods up to 70 min. These observations indicate that the endogenous TPN^+ in the homogenate is rate-limiting for GSSG reduction.

Both the aerobic and anaerobic rates of GSSG reduction increased in a non-linear fashion with TPN^+ concentration up to 0·11 μmole of added TPN^+/ml. (Table 5). The increase in the rate of GSSG reduction was about threefold with 0·0375, fourfold with 0·075 and 4·5-fold with 0·11 μmole of added TPN^+/ml.

When up to 0·11 μmole of TPN^+/ml. was added the aerobic and anaerobic rates of GSSG reduction were the same. When the TPN^+ concentration was raised above 0·11 μmole/ml. there was little change in the anaerobic rate of GSSG reduction, but the aerobic rate continued to increase. This resulted in a more rapid aerobic than anaerobic reduction of GSSG at high TPN^+ concentrations (Table 5). The TPN^+ concentration (about 0·11 mM) required to obtain maximum anaerobic rates of reduction of GSSG (Table 5, Expt. 1) is much higher than the concentration of reduced triphosphopyridine nucleotide (TPNH) (0·02 mM) found by McIlwain & Tresize (1957) to give maximum rates of GSSG reduction in brain extracts. This may be due to the fact that in liver homogenate the rate of reduction of TPN^+ limits the reduction of GSSG and possibly due to the destruction of added TPN^+.

Effect of citrate, fumarate and glucose 6-phosphate on the reduction of oxidized glutathione in pigeon-liver homogenates

When fumarate or citrate was added to the homogenate the rate of GSSG reduction was increased to about four times the endogenous rate (Table 6).

Table 4. *Effect of oxidized triphosphopyridine nucleotide on the time course of reduction of oxidized glutathione in pigeon-liver homogenates*

Vessels contained 0·2 ml. of approx. 0·05 M-GSSG (containing 9·0 μmoles of GSSG and 0·5 μmole of GSH), 3·0 ml. of homogenate; 0·1 ml. of 1·5 mM-TPN^+ was added, and phosphate saline to a total vol. of 4·0 ml.

Dry wt. of tissue (mg.)	TPN^+ added (μmole)	Endogenous GSH (μmole)	Initial GSH (μmoles)	Incubation time (min.)	Final GSH (μmoles)		GSH formed (μmoles)	
					N_2	O_2	N_2	O_2
50·5	Nil	0·8	1·2	10	1·9	2·0	0·7	0·8
	Nil			25	3·2	3·2	2·0	2·0
	Nil			70	5·2	4·5	4·0	3·3
	0·15			10	3·5	3·5	2·3	2·3
	0·15			25	5·4	5·6	4·2	4·4
	0·15			70	8·3	8·4	7·1	7·2
39·0	Nil	0·6	1·1	10	1·7	1·6	0·6	0·5
	Nil			25	2·1	2·0	1·0	0·9
	Nil			60	2·4	1·9	1·3	0·8
	0·15			10	2·8	2·8	1·7	1·7
	0·15			25	3·5	3·6	2·4	2·5
	0·15			60	4·0	3·8	2·9	2·7

Added TPN^+ (0·0375 mM) stimulated GSSG reduction three- to four-fold (Table 6). When both citrate and TPN^+ were added, GSSG reduction was stimulated 11-fold and 14-fold in Expts. 1 and 2 (Table 6) respectively, and in both experiments the stimulation by citrate plus TPN^+ was about equal to the product (stimulation by citrate alone × stimulation by TPN^+ alone). Glucose 6-phosphate (Table 6, Expt. 2) stimulated GSSG reduction 1·5 times and in the presence of added TPN^+ stimulated GSSG reduction sevenfold. The stimulation of GSSG reduction by glucose 6-phosphate plus TPN^+ was equal to the product (stimulation by TPN^+ alone × stimulation by glucose 6-phosphate alone). Thus the stimulatory effects of added TPN^+ and hydrogen donors are complementary, indicating that both factors are rate-limiting for the endogenous reduction of GSSG in the homogenate.

Citrate and fumarate are more than twice as effective as glucose 6-phosphate as hydrogen donors for the reduction of GSSG in pigeon-liver homogenate.

In Expt. 1 (Table 6) the aerobic rates of GSSG reduction with and without added citrate and fumarate were less than the anaerobic rates. However, when 0·15–0·45 μmole of TPN^+ was added (Tables 4–6), with or without additional substrate, the aerobic and anaerobic rates of reduction of GSSG were equal. This observation suggests that the aerobic rate of reduction of GSSG without added TPN^+ is lower than the anaerobic rate, owing to a more rapid breakdown of the coenzyme under aerobic conditions.

The maximum observed rate of GSSG reduction was 140 μmoles of GSSG/g. fresh wt. of tissue/hr., which is of the same order as the rate observed by

Table 5. *Effect of oxidized triphosphopyridine nucleotide concentration on the rate of reduction of oxidized glutathione in pigeon-liver homogenates*

In Expt. 1 the homogenate was prepared in phosphate saline and in Expt. 2 in bicarbonate saline. Vessels contained 0·2 ml. of approx. 0·05 M-GSSG (9·0 μmoles of GSSG + 0·5 μmole of GSH), 3·0 ml. of homogenate, 0·1–0·8 ml. of 1·5 mM-TPN^+, and phosphate (Expt. 1) or bicarbonate (Expt. 2) saline to a total vol. of 4·0 ml. Incubation time, 10 min. For further details see experimental section.

Dry wt. of tissue (mg.)	TNP^+ added (μmoles)	Endogenous GSH (μmoles)	Initial GSH (μmoles)	Final GSH (μmoles)		GSH formed (μmoles)	
				N_2	O_2	N_2	O_2
46·7	Nil	0·9	1·4	2·4	2·2	1·0	0·8
	0·15			4·4	4·4	3·0	3·0
	0·30			5·3	5·3	3·9	3·9
	0·45			6·0	5·8	4·6	4·4
	0·75			5·7	6·2	4·3	4·8
				N_2+CO_2	O_2+CO_2	N_2+CO_2	O_2+CO_2
73·2	Nil	1·3	1·9	4·4	4·4	2·5	2·5
	0·15			9·7	9·3	7·8	7·4
	1·20			9·9	12·2	8·0	10·3

Table 6. *Effect of citrate, fumarate and glucose 6-phosphate on the reduction of oxidized glutathione in pigeon-liver homogenates*

Vessels contained 0·2 ml. of approx. 0·05 M-GSSG (9·0 μmoles of GSSG + 0·5 μmole of GSH), 3·0 ml. of homogenate, 0·2 ml. of 0·1 M additional substrate; 0·1 ml. of 1·5 mM-TPN^+ was added and phosphate saline to a total volume of 4·0 ml. Incubation time, 10 min.

Expt. no.	Dry wt. of tissue (mg.)	Additional substrate	TPN^+ added (μmole)	Endogenous GSH (μmole)	Initial GSH (μmoles)	Final GSH (μmoles)		GSH formed (μmoles)	
						N_2	O_2	N_2	O_2
1	47·5	Nil	Nil	0·9	1·7	2·4	2·3	0·7	0·6
		Citrate	Nil			4·4	4·0	2·7	2·3
		Fumarate	Nil			4·4	4·0	2·7	2·3
		Nil	0·15			3·8	3·8	2·1	2·1
		Citrate	0·15			9·4	9·4	7·7	7·7
2	40·5	Nil	Nil	0·8	1·1	1·6	1·6	0·5	0·5
		Citrate	Nil			2·8	2·8	1·7	1·7
		Glucose 6-phosphate	Nil			1·9	1·8	0·8	0·7
		Nil	0·15			3·0	2·9	1·9	1·8
		Citrate	0·15			8·0	7·9	6·9	6·8
		Glucose 6-phosphate	0·15			4·8	4·6	3·7	3·5

Table 7. *Effect of pyruvate and fumarate on the reduction of oxidized glutathione in pigeon-liver homogenates in bicarbonate saline*

Vessels contained 0·2 ml. of approx. 0·05 M-GSSG (9·0 μmoles of GSSG + 0·5 μmole of GSH), 3·0 ml. of homogenate, 0·2 ml. of 0·1 M additional substrate as indicated below (except in Expt. 2, in which 0·2 ml. of 0·5 M-pyruvate was added), 0·1 ml. of 1·5 mM-TPN$^+$, and saline to a total volume of 4·0 ml. Incubation time, 10 min.

Expt. no.	Dry wt. of tissue (mg.)	Additional substrate	TPN$^+$ added (μmoles)	Endogenous GSH (μmole)	Initial GSH (μmole)	Final GSH (μmoles)		GSH formed (μmoles)	
						N_2 + CO_2	O_2 + CO_2	N_2 + CO_2	O_2 + CO_2
1	73·2	Nil	Nil	1·3	1·9	4·4	4·4	2·5	2·5
		Pyruvate	Nil			3·3	4·4	1·4	2·5
		Fumarate	Nil			7·0	6·9	5·1	5·0
		Nil	0·15			9·7	9·3	7·8	7·4
		Pyruvate	0·15			9·2	10·4	7·3	8·5
2	64·5	Nil	Nil	1·2	1·8	4·5	4·4	2·7	2·6
		Pyruvate	Nil			2·5	2·6	0·7	0·8
		Fumarate	Nil			6·7	6·5	4·9	4·7
		Pyruvate + fumarate	Nil			3·1	4·2	1·3	2·4
		Nil	0·15			9·7	9·8	7·9	8·0
		Pyruvate	0·15			6·6	6·6	4·8	4·8
3	35·4	Nil	Nil	0·4	0·6	1·1	1·0	0·5	0·4
		Pyruvate	—	—	0·7	0·9	1·0	0·2	0·3

McIlwain & Tresize (1957) for cerebral tissue (250–300 μmoles of GSSG/g. fresh wt. of tissue/hr.). Higher rates could probably be obtained by increasing the concentration of added TPN$^+$.

Effect of pyruvate and fumarate on the reduction of oxidized glutathione in pigeon-liver homogenates in bicarbonate saline

In order to ascertain if a mechanism other than coupling to TPN-linked oxidation (see Discussion) is involved in the aerobic reduction of GSSG, experiments were carried out under conditions similar to those used by Krebs (1954) and L. Rathbone & H. A. Krebs (unpublished work, 1959) in studies of malate formation. Homogenates were prepared in bicarbonate saline (for details see Materials and Methods section), manometers were gassed with oxygen–carbon dioxide or nitrogen–carbon dioxide and the reduction of GSSG was studied in the presence of high concentrations (25 mM) of pyruvate (Table 7, Expt. 2) similar to those employed by L. Rathbone & H. A. Krebs (unpublished work, 1959). No stimulation of GSSG reduction by oxygen was, however, observed.

Pyruvate at low concentrations (5 mM) inhibited the anaerobic reduction of GSSG by about 50 %, but had no effect under aerobic conditions (Table 7). Under the same conditions fumarate (5 mM) stimulated both the aerobic and anaerobic reduction of GSSG about twofold (Table 7). When pyruvate and TPN$^+$ were added together the anaerobic reduction of GSSG was inhibited by only 7 %. With higher concentrations of pyruvate (25 mM), similar to those used by L. Rathbone & H. A. Krebs (unpublished work, 1959), both the aerobic and the anaerobic reduction of GSSG were inhibited by about 70 % (Table 7, Expt. 2). When pyruvate (25 mM) and TPN$^+$ were added together the reduction of GSSG aerobically and anaerobically was about 40 % less than with TPN$^+$ alone. With pyruvate (25 mM) and fumarate (5 mM) the aerobic reduction of GSSG was restored to its endogenous rate and the anaerobic reduction of GSSG was increased to about half of its endogenous rate.

Thus fumarate partially counteracts the inhibition of GSSG reduction by pyruvate, being more effective under aerobic than anaerobic conditions. Alternatively pyruvate may be considered to reverse the stimulation of GSSG reduction by fumarate.

It is possible that the reductive carboxylation of pyruvate catalysed by malic enzyme (Ochoa, Mehler & Kornberg, 1948) competes with glutathione reductase for TPNH and hence inhibits the reduction of GSSG. Why low concentrations (5 mM) of pyruvate inhibit GSSG reduction anaerobically but not aerobically is not clear. Perhaps pyruvate is rapidly metabolized under aerobic conditions by pathways other than the malic enzyme reaction, and hence does not compete with GSSG for TPNH. When a large excess of pyruvate is present aerobically it could participate in the malic enzyme reaction as well as being metabolized by other pathways and hence compete with GSSG for TPNH. The finding that fumarate partially counteracts the inhibitory action of pyruvate supports these ideas, since fumarate is a precursor of malate, which would be expected to inhibit the reductive carboxylation of pyruvate by mass action.

DISCUSSION

The striking differences in the stability of GSH and the rate of reduction of GSSG in homogenates of pigeon, rat and guinea-pig liver (Tables 1 and 2) does not necessarily reflect the behaviour of GSSG in the initial tissue. This is suggested by the fact that the glutathione of a variety of intact tissues was found by Bhattacharya *et al.* (1955) to be predominantly in the reduced form.

Thus in the intact respiring tissue the rate of reduction of GSSG must be rapid enough to counteract the oxidation of GSH. The nature of the changes which increase the rate of oxidation of GSH and decrease the rate of reduction of GSSG, or both, on homogenization of rat liver require further study. Pigeon-liver homogenate maintained both endogenous and added GSH in the reduced form when incubated aerobically for periods of more than 1 hr. (Table 1) and was therefore assumed to be more similar to the intact tissue than the rat-liver or guinea-pig-liver homogenate. Hence pigeon-liver homogenate was chosen for further investigations.

When GSH was incubated with boiled tissue at 30° it was oxidized at the rate of about 17 %/hr. (Table 1). The concentration of endogenous GSH in pigeon-liver homogenate is about $4{\cdot}6 \pm 0{\cdot}4$ μ-moles/g. fresh wt. of tissue/hr. (12 expts.) and therefore about 0·8 μmole (17 %) of the endogenous GSH could be oxidized non-enzymically/g. fresh wt. of tissue/hr. The initial rates of reduction of added GSSG in pigeon-liver homogenate were $13{\cdot}8 \pm 5{\cdot}0$ μmoles of GSSG/g. fresh wt. of tissue/hr. (9 expts.), which is high enough to maintain the endogenous GSH in the reduced form if we assume there is no enzymic GSH oxidation. However, enzymic oxidation possibly exists and more than 0·8 μmole of GSH is oxidized. By supplementing pigeon-liver homogenate with TPN^+ and citrate, rates of reduction as high as 140 μmoles of GSSG/g. fresh wt. of tissue/hr. were obtained. Krebs (1954) observed that the formation of malate from pyruvate and carbon dioxide in pigeon-liver homogenates is more rapid under aerobic than under anaerobic conditions. Since coupling of the reductive carboxylation of pyruvate to TPN-linked oxidations should be just as effective anaerobically as aerobically, Krebs suggested that some mechanism other than simple coupled oxidation–reduction was involved in the aerobic formation of malate. He proposed that aerobically, when the ratio adenosine triphosphate/adenosine diphosphate (ATP/ADP) was high, TPNH could be formed by a reversal of the first step of oxidative phosphorylation according to the equation:

$$\text{Dihydroflavoprotein} + TPN^+ + ATP \rightarrow \text{Flavoprotein} + TPNH + ADP + \text{phosphate} + H^+$$

If such a mechanism were to act in pigeon liver one would expect TPN-linked reductions generally to be more rapid aerobically than anaerobically. However, under most conditions the aerobic and anaerobic rates of reduction of GSSG were approximately equal. It is interesting, though, that added DPN^+ at concentrations above 0·11 mM has a stimulating effect on GSSG reduction, higher under aerobic than anaerobic conditions (Table 5). This suggests an effect of oxygen on GSSG reduction, perhaps by a conversion of TPN^+ into TPNH by a mechanism other than TPN-linked oxidations.

In the presence of 5 mM-pyruvate the aerobic reduction of GSSG was more rapid than the anaerobic reduction (Table 7), but this may be possibly due to an inhibition of the anaerobic rate by pyruvate and not to a stimulation by oxygen. The aerobic rate of reduction of GSSG was up to 30 % higher than the anaerobic rate when large quantities of TPN^+ (more than 0·45 μmole/flask) were added (Table 5). However, both these effects were small in comparison with the large (up to ten-fold) difference between the aerobic and anaerobic formation of malate from pyruvate and carbon dioxide (Krebs, 1954; L. Rathbone & H. A. Krebs, unpublished work, 1959). It is possible that the low value for GSSG reduction by oxygen that was observed is related to swelling of homogenate mitochondria caused by GSH added or formed from the added GSSG (Lehninger & Schneider, 1959).

Although both GSSG reduction and reductive carboxylation of pyruvate are higher in aerobic than in anaerobic conditions, we cannot say whether they have the same origin.

SUMMARY

1. There was no disappearance of reduced glutathione incubated aerobically with pigeon-liver homogenate, but with rat-liver homogenate 36 % of the initial reduced glutathione disappeared in 1 hr. at 30°. On incubation with boiled rat-liver or pigeon-liver homogenate 17 % of the initial reduced glutathione disappeared in 1 hr.

2. In homogenates of rat and guinea-pig liver the anaerobic rate of reduction of oxidized glutathione was more rapid than the aerobic rate. The aerobic reduction of oxidized glutathione in the first 10 min. was followed by reoxidation of the reduced glutathione formed. In pigeon-liver homogenates the initial (0–20 min.) aerobic and anaerobic rates of reduction of oxidized glutathione were approximately equal.

3. Addition of oxidized triphosphopyridine nucleotide (final concentration up to 0·11 mM) to

pigeon-liver homogenates stimulated the aerobic and anaerobic rates of reduction of oxidized glutathione to the same degree. At concentrations of oxidized triphosphopyridine nucleotide above 0·11 mM the anaerobic reduction of oxidized glutathione was lower than the aerobic reduction.

4. Citrate, fumarate and glucose 6-phosphate stimulated oxidized glutathione reduction in pigeon-liver homogenates. The addition of oxidized triphosphopyridine nucleotide (0·04 mM) further stimulated the reduction.

5. In pigeon-liver homogenates incubated under 5% carbon dioxide, 5 mM-pyruvate inhibited the anaerobic reduction of oxidized glutathione by about 50% but had no effect aerobically. Higher concentrations (25 mM) of pyruvate inhibited both the aerobic and anaerobic reduction of oxidized glutathione by about 70%. Added fumarate and oxidized triphosphopyridine nucleotide both partially counteracted the inhibition of oxidized glutathione reduction due to pyruvate. Fumarate was more effective in aerobic conditions.

6. The significance of these findings is discussed in relation to the mechanism of maintenance of glutathione in the reduced form in intact tissue.

The author would like to thank Professor Sir Hans Krebs, F.R.S., for suggesting this problem and for his encouragement, advice and criticism, Dr R. G. Kulka for his constant help as supervisor, Dr W. Bartley for valuable discussions and Dr R. Cecil for instruction in SH-group analysis. He would also like to thank the Calouste Gulbenkian Foundation for a fellowship and The Distillers Co. Ltd. (Biochemicals) for a gift of GSH. This work was aided by a grant from the Rockefeller Foundation.

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The Pectic Enzymes of *Aspergillus niger*

1. THE PRODUCTION OF ACTIVE MIXTURES OF PECTIC ENZYMES

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Aspergillus niger, like many other fungi, produces enzymes which attack pectic substances. It is probably true to say that none of these enzymes has been obtained in a pure state from a filamentous fungus, although Patel & Phaff (1959) produced preparations of a single pectic enzyme from the yeast *Saccharomyces fragilis*. Some work has been published on the mode of action of mould enzymes (Jansen & MacDonnell, 1945; Saito, Yasuji & Marumo, 1954; Ozawa & Okamoto, 1954; Ayres, Dingle, Phipps, Reid & Solomons, 1952; Saito, 1955), but almost invariably crude preparations were used, often obtained from commercial material of unknown provenance; where defined culture methods were employed the activities reported were very low (Saito, 1955) in comparison with those of the commercial preparations.

The entire field of pectic enzymes is extremely confused; almost as many systems of nomenclature exist as authors who have dealt with this subject. A recent review by Demain & Phaff (1957) has brought a welcome clarification and we have adopted their system of nomenclature. Basically this scheme divides pectic enzymes into poly-

methylgalacturonases, which preferentially attack pectin rather than pectic acid, and polygalacturonases, which preferentially attack the latter. Those enzymes which have a terminal mechanism of attack are distinguished by the prefix 'exo' and those with a random mechanism of attack by the prefix 'endo'.

Little information is available on culture methods employed for the commercial production of pectic enzymes. Some of these are apparently produced on solid bran cultures (Swangard, 1947) and the mycelium is often mechanically disintegrated and then extracted to obtain intracellular enzymes. Most laboratory experiments have been made with either surface cultures or shake-flasks (Brooks & Reid, 1955; Saito, 1955; Takehana & Ogura, 1955). Brooks & Reid (1955) reported that *Aspergillus foetidus* produced both endopolygalacturonase and exopolygalacturonase in surface cultures but only endopolygalacturonase in submerged cultures. We have concentrated primarily on the production of the enzymes by submerged growth in stirred fermenters of 10 l. nominal capacity.

Saito (1955) investigated factors affecting the production of pectic enzymes in shake-flask cultures of *A. niger* and reported that endopolygalacturonase (or 'depolymeric polygalacturonase') was constitutive whereas the exopolygalacturonase (or 'galacturonogenic polygalacturonase') was adaptive to the presence of pectic substances. Kontio (1950) reported that the 'pectase' of *A. niger* was adaptive to the presence of pectin.

The present work has had two principal objectives: first, to produce relatively large quantities of crude mixtures of enzymes of high pectolytic activity from a single mould grown under reproducible conditions; this part of the work is described in the present paper; secondly, to separate and purify as far as possible the individual enzymes present in this mixture of known provenance, in order to determine their precise roles in the breakdown of pectin. The next paper describes the purification and properties of endopolygalacturonase.

METHODS AND MATERIALS

Biological techniques

The concentrations of solutions given as percentages refer to w/v throughout.

Selection of strains of micro-organisms. Although the ability to produce pectic enzymes is widespread amongst micro-organisms, it was necessary to screen many of them to obtain highly active strains. Organisms were obtained from a wide variety of sources: from the air, from soil, from grape-must and from beer exposed to the air; special attention was given to infected and decaying vegetable material such as apples, oranges, tangerines, pears and carrots, whose high pectin content might be expected to encourage pectolytic organisms. The majority of the organisms studied were moulds belonging to the genera *Aspergillus, Penicillium, Sclerotinia, Fusarium* and *Mucor*, all of which are well known as producers of pectic enzymes.

Whenever possible, spores were taken directly from the infected material to bean–sucrose agar (Tanner, 1919). Otherwise the required organism was separated by plating on 2% Czapek (1902) agar in which the sucrose had been replaced by 2% pectin. After incubation at 30°, samples of pure culture were transferred to slants of bean–sucrose agar and incubated at 30° for 6–7 days.

Rapid screening of organisms for pectolytic activity was achieved by inoculating them into tubes containing 9 ml. of a 5% solution of pectin in a 2% aqueous extract of groundnut meal, at pH 4·2. In the presence of pectolytic strains the medium rapidly lost its initially high viscosity, and the turbid material in the medium gradually precipitated, leaving a clear zone at the top of the tube. This zone increased in extent with time, and the rate of this increase proved to be a good indication of the pectolytic activity of the organism.

Those strains which proved to be highly pectolytic were further screened by testing the activities of their culture fluids when grown in shake-flasks on the routine sucrose-pectin medium described below; the results of the two screening methods were in good agreement.

Storage of micro-organisms. Cultures of the organisms were preserved on bean–sucrose agar, or as conidia on soil or barley cultures, held at 4°.

Preparation of spore suspensions for inoculation. Spore suspensions were prepared by adding 5 ml. of conidial suspension to 250 ml. Erlenmeyer flasks containing 15 g. of pearl barley moistened with 5 ml. of a sporulation solution (asparagine, 0·1%; glycerol, 3%; Whiffen & Savage, 1947). The flasks were incubated at 30° for a week and then stored at 4°. When required for use, 20 ml. of a sterile 0·01% solution of lauryl sulphate was added followed by 100 ml. of water, and the conidial suspension obtained was passed through a sterile cotton-wool filter. The concentration of the suspension was determined with a Thoma counting chamber.

Shake-flask fermentations. Preliminary experiments were performed in 500 ml. flasks containing 100 ml. of medium agitated in a rotary shaker (Paladino, 1954) at 30°. The flasks were seeded with 1 ml. of a suspension of about 10^7 spores. Fermentations were continued for 5 days at 30°.

Stirred fermenters. Larger-scale fermentations were performed in 10 l. stainless-steel stirred fermenters (Chain, Paladino, Ugolini & Van der Sluis, 1954). After the media within the fermenters were sterilized with steam at atmospheric pressure for 20 min. followed by steam at 110° for 30 min., the fermenters were brought to 30°. The fermenters were fitted with stirrers of 90 mm. diameter, bearing eight vertical blades and rotating at 750 rev./min. Air was admitted at 1 atm. overpressure with a flow rate of 5 l./min.

Sterility tests. Samples withdrawn periodically from the fermenters were tested for microbial contamination by inoculation into liquid and solid media.

Ethanol precipitations. The details of the technique used for ethanol precipitation of the enzyme are described in the

next paper. The precipitate obtained (stage 1, Mill & Tuttobello, 1961) was usually dissolved in ice-cold water in a Waring Blendor, centrifuged and freeze-dried.

Analytical procedures

Evaluation of enzymic activity. No attempt was made at this stage to individualize the various pectic enzymes, but a number of criteria were applied to characterize the ability of the various culture fluids and enzyme preparations to digest pectin.

Changes in the viscosity of pectin solutions were followed in 5 ml. Ostwald viscometers at 30°. A 2·5% solution of pectin was prepared in 1% NaCl and the pH adjusted to 4·0; since considerable difficulty was often encountered in obtaining a clear solution, this was centrifuged and diluted with water so that when 1 ml. was diluted with 4 ml. of 0·2M-acetate buffer, pH 4·0, the resultant mixture had a relative viscosity of 3·5. In use 1 ml. of the 2·5% pectin solution was mixed with 4 ml. of a suitable dilution of the enzyme in the acetate buffer and the fall in viscosity measured. The time required to reduce the relative viscosity by half (i.e. to 1·75) was inversely proportional to the enzyme concentration (Fig. 1). One unit of activity was taken as the amount of enzyme which under the conditions defined reduced the relative viscosity to 1·75 in 20 min. The preparation used for Fig. 1 was, of necessity, simply the crude culture fluid of our earliest preparations. The enzyme content was so low in comparison with other nitrogenous components that neither nitrogen content nor extinction would have much true significance. The solutions used for the test were simply dilutions of the culture fluid obtained in a particular experiment and hence served merely to establish the essential linearity of the assay response without having any absolute significance.

The viscosity-diminishing assay tends to measure preferentially the 'endo' activity of the enzymic mixtures (Demain & Phaff, 1957), so that the preparations obtained were also tested for their ability to release reducing groups from pectin and for the extent to which the pectin was digested. One part of the culture fluid was mixed with 9 parts of a 2% solution of pectin at pH 4; a little toluene was added and the mixture was incubated at 30°. Samples were withdrawn at intervals and assayed by the method of Somogyi (1952).

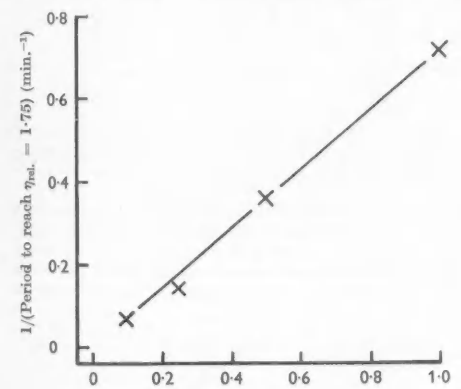

Fig. 1. Activities of various concentrations of pectolytic enzymes in diminishing the viscosity of a solution of pectin from $\eta_{rel.}$ = 3·5 to 1·75, at pH 4·0 and 30°.

Dry weights. Dry weights were determined by filtering 100 ml. of the culture through a layer of cotton wool and gauze, washing the mycelium with water and drying at 100°.

Sucrose consumption. The concentration of sucrose and invert sugar remaining in the culture medium during the fermentation was determined by heating centrifuged samples in the presence of 2N-HCl at 100° for 20 min., followed by the determination of the fructose present by the resorcinol method (Roe, 1934); this technique will of course also measure the fructose present in the culture medium, so that, if there were a preferential utilization of either the glucose or fructose moieties of the sucrose, an erroneous picture of the sugar consumption would be obtained. However, chromatographic studies showed that the concentrations of free glucose and fructose in the culture fluid remained approximately equal throughout the fermentation.

Chromatography. Chromatograms were run on Whatman no. 541 paper with, as solvent, the epiphase of *iso*butyric acid–acetic acid–water (200:1:200) (Mill & Tuttobello, 1961); sugars were revealed by the $AgNO_3$-dip technique (Trevelyan, Procter & Harrison, 1950).

pH *measurements.* These were made with a glass electrode.

Chemicals

Pectin. Apple pectin (Fluka, A. G., Buchs, S. G., Switzerland) with a uronic anhydride content of about 81% (by the method of McCready, Swenson & Maclay, 1946) was used.

Groundnut-meal extract. A 2% suspension of defatted groundnut meal (containing 47–49% of protein and 0·5–1% of lipid) in water was boiled for 30 min. with continuous stirring, cooled, filtered through muslin and adjusted to pH 7·4 with N-NaOH.

Buffers. Buffers were formulated according to Gomori (1955) from analytical-grade reagents.

Antifoam agent. An 8% solution of Alkaterge C (Commercial Solvents Corp., U.S.A.) in seed oil was added in the proportion of 3 ml./l. of medium before sterilization.

RESULTS

Selection of a suitable strain of mould. Of the many organisms screened for their ability to produce pectic enzymes, *A. niger* strain CH, which had been isolated from decaying fruit, was the most active and was used for all the subsequent work.

Effects of the composition of the culture medium. Various media were tried initially in shake-flask cultures. Two types of media were first tested: synthetic media based on those of Czapek (Czapek, 1902), Czapek–Dox (Clutterbuck, Lovell & Raistrick, 1932), Jarvis–Johnson (Jarvis & Johnson, 1947) and Shu–Johnson (Shu & Johnson, 1948),

modified in each case by the replacement of their carbohydrate by 2 % of pectin plus 2 % of sucrose; and more complex media with organic sources of nitrogen. Five of the latter were employed [the groundnut extract; a 2 % suspension of groundnut meal; a 2 % suspension of soya meal; a 2 % corn-steep liquor; and a nutritive broth (1 % of meat extract and 1 % of peptone)], and in each case 0·2 % of NH_4NO_3 and 0·05 % of Na_2SO_4 were added, the pH was adjusted to 7·4 and then 4 % of a mixture of equal parts of pectin and sucrose was stirred in. After sterilization the pH of each mixture fell to about 4.

There was a marked difference in the appearances of the cultures produced on the two classes of media. In the synthetic media the growth of the mycelium was scanty and in the form of loose fluffy pellets; in the media with organic sources of nitrogen the growth was very much heavier, being mainly filamentous in those media containing suspensions of the meals, and consisting of large numbers of small, dense and compact pellets in the others.

This morphological difference was accompanied by a similarly marked difference in the activity of the culture fluids measured by the diminution of the viscosity of pectin solutions. Fig. 2 shows the development of this activity during the fermentation. The cultures in the synthetic media all yielded only feeble activity and only the most active (that based on Czapek–Dox medium) is shown in the Figure. It is clear that the medium containing the groundnut-flour extract is superior to the others tested.

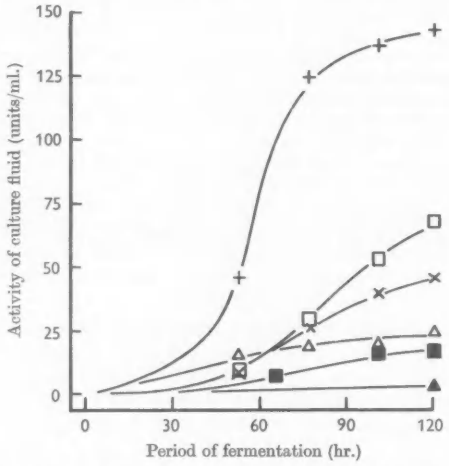

Fig. 2. Development of pectin-viscosity-diminishing activity in shake-flask cultures of *A. niger* grown at 30°. The media were: (1) ▲, Czapek–Dox medium containing 2 % of sucrose and 2 % of pectin; (2) 2 % of sucrose, 2 % of pectin, 0·05 % of Na_2SO_4 and 0·2 % of NH_4NO_3 in: ■, 2 % corn-steep; △, 2 % soya flour; ×, 2 % groundnut-meal suspension; □, nutrient broth; +, groundnut-meal extract.

The effects of variations in the carbohydrate content were tested in a similar series of experiments. In each case the media contained 0·05 % of Na_2SO_4 and 0·2 % of NH_4NO_3 in the groundnut-flour extract; various carbohydrates were added and the activities obtained after 5-days' growth in shake-flasks are shown in Table 1. Experiments performed in stirred fermenters with the same media gave cultures with much higher activities; the relative efficiencies of the media proved to be very similar to those shown in the shake-flasks, except that galactose was no more effective than sucrose. In view of its low cost the latter was selected for further experiments. Fermentations were performed in tanks with the same basic medium but with variations in the relative amounts of sucrose and pectin used; the total carbohydrate content was maintained at 4 %. Table 2 reports the

Table 1. *Effects of changes in the carbohydrate content of the medium on the viscosity-diminishing activity of the culture fluid*

The media contained the carbohydrates together with 0·02 % of Na_2SO_4 and 0·5 % of NH_4NO_3 in groundnut-flour extract. Fermentations were carried out in shake-flasks for 5 days.

Carbohydrate content of medium (%)	Activity of culture fluid (units/ml.)
None	24
Pectin (2) + starch (3)	28
Pectin (2) + dextrin (3)	88
Pectin (2) + fructose (3)	100
Pectin (2) + sucrose (3)	101
Pectin (2) + glucose (3)	101
Pectin (2) + galactose (3)	159
Pectin (5)	100

Table 2. *Effect of changes in the pectin and sucrose contents of the medium on the viscosity-diminishing activities of the culture fluids*

The media contained the carbohydrates together with 0·02 % of Na_2SO_4 and 0·5 % of NH_4NO_3 in groundnut-flour extract. Fermentations were carried out in stirred fermenters for 5 days. The extent of digestion of pectin was measured with a 1 % soln. of pectin containing 1 % of the ethanol-precipitated enzymes.

Carbohydrate content of medium (%)	Activity of culture fluids (units/ml.)	Digestion of pectin (% of complete hydrolysis)
Pectin (4)	90	100
Pectin (3) + sucrose (1)	160	100
Pectin (2) + sucrose (2)	210	95
Pectin (1) + sucrose (3)	125	88
Sucrose (4)	80	85

activities obtained in terms of the pectin-viscosity-diminishing activities of the culture fluids and gives the extent to which a solution of pectin is digested after prolonged incubation with the enzymes obtained from these fluids by ethanol precipitation followed by freeze-drying. Even with the high concentration of enzyme used (1 %), digestion did not go to completion with the enzymes obtained from growth on the media containing little or no pectin; when the original culture fluid was used directly as the enzyme, only some 60 % digestion could be produced with the culture obtained in the absence of pectin. With pectin as the sole carbohydrate source, the culture fluid induced complete hydrolysis of pectin.

The combination of 2 % of sucrose and 2 % of pectin was used as the normal medium.

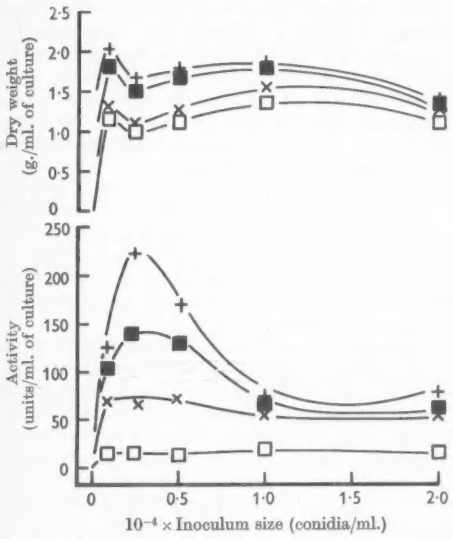

Fig. 3. Effect of inoculum size on the development of pectin-viscosity-diminishing activity and dry weight in 5 l. stirred fermentations in the pectin–sucrose–groundnut-extract medium. Measurements were made after 40 (□), 80 (×), 100 (■) and 120 (+) hr. of growth.

pH *during the fermentation.* In a typical fermentation in the pectin–sucrose medium, the pH fell slightly from its initial value of about 4 during the first 24 hr. and then remained fairly constant at about 3 for a further 5 days, when it once more rose to about 4. With pectin as the sole carbohydrate present, it rose rather more rapidly, whereas with sucrose alone it often fell below pH 2. Marked changes of pH during the fermentation led to a loss of activity. With the pectin–sucrose

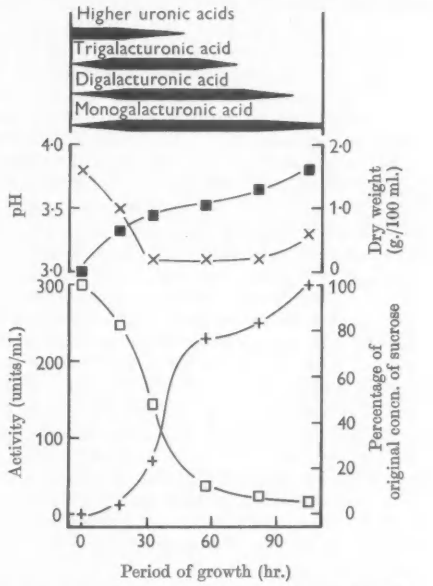

Fig. 4. Progress of a typical fermentation performed as described in the text. +, Pectin-viscosity-diminishing activity; □, relative sucrose concentration; ■, mycelial dry weight; ×, pH. The horizontal bars at the top of the diagram represent the presence in the medium of galacturonic acid polymers derived from the pectin; these uronic acids were detected chromatographically, and the width of the bars is intended to convey a subjective estimate of the relative intensities of the chromatographic spots as judged by eye.

Table 3. *Comparison of the activities of enzymes from different sources*

Enzyme		Viscosity-diminishing activity (units/mg.)
Preparation	Source	
Stage 1 (batch 42)	—	2·5
Pectasin 100-D Pectinol (soluble, double concn.)	Rohm and Haas Co., Philadelphia, 5, Pa., U.S.A.	0·25 0·01
Pectinase	General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A.	0·25
Carterzyme	H. W. Carter and Co. Ltd., Coleford, Glos.	0·67

medium there was no need to correct the pH during the fermentation and this was a further advantage of this combination.

Effects of inoculum size. During these experiments it became clear that the direct inoculation of the fermenters with a suspension of conidia was a more suitable and convenient technique than the use of mycelial inocula; however, the size of the inoculum had a pronounced effect on the activity of the culture obtained. Fig. 3 shows the development of pectin-viscosity-diminishing activity, and changes in dry weight in cultures in 10 l. fermenters inoculated with 2×10^4 to 2×10^5 spores/ml. of medium. It is apparent that an optimum inoculum size exists which under these conditions was 4×10^4 to 5×10^4 spores/ml. of medium.

A typical fermentation. In a 10 l. stirred fermenter were placed 4·8 l. of the groundnut-flour extract containing 0·2 % of NH_4NO_3 and 0·05 % of Na_2SO_3, adjusted to pH 7·4–7·5. A mixture of 100 g. of pectin and 100 g. of sucrose was now added slowly with vigorous stirring to prevent the formation of lumps, followed by 15 ml. of antifoam agent. After being sterilized and cooled, the fermenter was seeded with 2×10^8 spores. It proved to be convenient to stop the fermentation after the fifth day; little increase in activity was obtained with longer periods. The culture was quickly filtered under vacuum through a thin layer of cotton wool or gauze on a wire mesh. The filtrate was preserved at 4° in the presence of a little toluene and as soon as possible purified to stage 1 (Mill & Tuttobello, 1961) and freeze-dried.

Fig. 4 shows the progress of such a fermentation in terms of the production of pectin-viscosity-diminishing activity, the change in pH, the consumption of sucrose and the presence in the culture of those oligogalacturonides, derived from the pectin, which can be detected chromatographically.

Table 3 compares the activity of the final product with that of various commercial preparations. Two to three grams of this product were obtained from each litre of fermentation fluid.

DISCUSSION

The composition of the fermentation medium had a decisive effect on both the nature and quantity of the pectic enzymes produced by *A. niger*. The synthesis of 'endo' enzymes (measured by the viscosity-diminishing assay) even in the absence of pectic substances shows that they are constitutive; the enzymes produced certainly included endopolygalacturonase, but it is not yet possible to say whether endopolymethylgalacturonase is also present. Nevertheless, the addition of pectin to the medium markedly increased the 'endo' activity. It is characteristic of the endopolygalacturonase that the digestion of pectin, or rather of pectic acid, does not go to completion (Demain & Phaff, 1957; Mill & Tuttobello, 1961). The preparations obtained in the presence of sucrose as sole carbon source in fact tended to produce only a limited hydrolysis, whereas those obtained with a pectin-containing medium induced virtually complete hydrolysis; presumably the latter preparations included also the 'exo' enzymes. The use of a mixture of sucrose and pectin as the carbohydrate source was justified for the higher activities obtained with the mixture than with either component alone, for the reduction in cost compared with the use of pectin by itself and for the increased pH stability during the fermentation, which obviated the need for pH control.

The efficacy of carbohydrates other than sucrose varied. In shake-flasks some difference was found between the different sugars, but these virtually disappeared with cultures in stirred fermenters. However, polysaccharides were markedly less effective than the mono- and di-saccharides tested.

Lullar & Johar (1953) reported that the addition of lucerne powder to their media increased the yield of pectic enzymes from *Penicillium notatum*. Various proteinaceous substances have a similar effect with *A. niger*. The greatest effect was found with the boiled extract of groundnut meal, which increased the viscosity-diminishing activities of the cultures by as much as 50-fold in comparison with cultures on the media containing only carbohydrates and salts.

These results are not in agreement with those of Saito (1955) for shake-flask cultures of a strain of *A. niger*. This author traced a general relationship between the amount of carbohydrate present in the culture and the amount of enzyme produced, irrespective of the nature of the carbohydrate. Our experiments, however, have shown merely that the system is exceedingly complex and that the most that can reasonably be expected is to discover empirically a particular set of conditions which will give a high yield of enzyme. The production of the pectic enzymes is probably dependent to a large extent on the general metabolic status of the culture. Under the present conditions there is a certain synergism of pectin and sucrose, which leads to the highest pectolytic activity being obtained when the two carbohydrates are present in similar concentrations. It is probable that changes in other factors such as the nitrogen source would also lead to changes in the optimum carbohydrate proportions.

The production of pectic enzymes is not proportional to the amount of mycelium produced in the culture. The experiment on inoculum size demonstrates that maximum enzymic activity is

obtained with an inoculum size which does not give maximum mycelial dry weight. The pectic enzymes show a marked increase during the third day of growth, and this point is marked also by the virtually complete utilization of the sucrose originally present in the culture and by a discontinuity in the curve of mycelial dry weight.

SUMMARY

1. A method is described for the preparation of a mixture of pectic enzymes with cultures of *Aspergillus niger*.

2. The cultures were produced in stirred fermenters with a medium consisting of 0·05 % of Na_2SO_4, 0·2 % of NH_4NO_3, 2 % of sucrose and 2 % of pectin in a boiled extract of groundnut flour. Fermentation was continued for 5–6 days at 30°. The pH of the culture remained between 3 and 4.

3. The preparations induced virtually complete hydrolysis of pectin.

4. Enzymic preparations obtained from the culture fluid by precipitation with ethanol were more active, on a weight for weight basis, than commercially available materials.

We wish to acknowledge the award of research fellowships by the Istituto Superiore di Sanità. We are grateful to the Direction and staff of the pilot plant of the Institute for the provision of facilities and technical assistance. We would especially like to recognize the encouraging guidance and help of Professor E. B. Chain, F.R.S.

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The Pectic Enzymes of *Aspergillus niger*

2. ENDOPOLYGALACTURONASE

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Endopolygalacturonase, as defined by Demain & Phaff (1957), is an enzyme hydrolysing the galacturonosidic links of pectic substances. It acts preferentially on pectic acid rather that on pectin in contrast with endopolymethylgalacturonase; the mechanism of attack is random and does not appear to go to completion, in contrast with exopolygalacturonase with its terminal mechanism of attack. Saito (1955) attempted to purify this enzyme from *Aspergillus niger* under the name of depolymeric galacturonase, but he nowhere expresses the activities of his preparations in terms of their protein content and as no criteria are given for the purity of the final preparation it is not

possible to say to what extent the purification was successful.

McCready & Seegmiller (1954) purified an endopolygalacturonase from a commercial enzyme preparation, Pectinol, whose source is unknown but presumably fungal. These authors tested the ability of their preparation to hydrolyse purified oligogalacturonic acids. The preparation acted against all those oligogalacturonic acids tested, including the dimer and trimer, but here the action was feeble. It was concluded that this endopolygalacturonase would hydrolyse any $(1 \rightarrow 4)$-α-link between two galacturonic residues with free carboxyl groups.

These results are largely in accordance with those obtained by Patel & Phaff (1959) with a well defined endopolygalacturonase obtained from the culture fluid of *Saccharomyces fragilis*, except that this enzyme did not attack digalacturonic acid.

In a definitive study of this enzyme from a particular mould it is therefore necessary to purify the material as extensively as possible, so as to exclude the catalysis of reactions by traces of exopolygalacturonase contaminating the preparation of endopolygalacturonase. This paper describes the partial purification of an endopolygalacturonase from culture fluids of *A. niger* in good yield and with a purification of more than 400-fold, and outlines some of the properties of this preparation, which does not hydrolyse digalacturonic acid.

EXPERIMENTAL

Methods and materials

Fermentations. The techniques employed for the fermentative production of a mixture of pectic enzymes are described in the preceding paper (Tuttobello & Mill, 1961).

A. niger strain CH, which had been isolated from decomposing fruit, was used throughout.

Ethanol precipitations. These were carried out in vessels of 20 and 50 l. capacity provided with jackets, through which a refrigerant mixture was circulated. Ethanol (95%), precooled to $-20°$, was added slowly to the mixture from large tap-funnels. Efficient stirring was maintained throughout and the temperature was held as close to the freezing point of the mixture as possible, without the actual formation of ice, down to a minimum of $-10°$. Centrifuging was performed with a Sharples air-driven centrifuge fitted with a cooling coil.

Pectin and pectic acid. Apple pectin (Fluka A. G., Buchs, S. G., Switzerland) was used after being washed with 80% ethanol containing 0·05 N-HCl; it had a uronic anhydride content of 81% (by the method of McCready, Swenson & Maclay, 1946) and 7·6% of methoxyl (by the method of Myers & Baker, 1934).

Pectic acid was produced from this by alkaline saponification (Kertesz, 1951) and was precipitated three times with 60% ethanol. The precipitated gel was redissolved in water with the cautious addition of N-NaOH to produce a pH of 4·0. After centrifuging, the solution was freeze-dried and the product stored in a desiccator. The dry product contained 83% of uronic anhydride. A portion (2 g.) of this product was dissolved in water containing 0·5% of disodium ethylenediaminetetra-acetate, the pH was adjusted if necessary, and then the solution was diluted to a volume of 100 ml. This constituted the solution designated as '2% sodium pectate'. It was dispensed in test tubes in 5 ml. quantities and held at $-20°$ until required; any of the thawed solution not used in the same day was discarded. A 1% solution of the sodium pectate at pH 4·8 and 30° had a relative viscosity of 6·0–6·5.

Separation of oligogalacturonides. Mixtures of oligouronides were produced by the digestion of pectin with fungal-pectinase preparations: the progress of the reaction was followed chromatographically and it was stopped by heating when a suitable composition had been reached. The bulk of the galacturonides, other than the monomer, were precipitated as their strontium salts (Luh & Phaff, 1954). The free acids, generated by treatment with Amberlite IR-120 (H^+) cation-exchange resin, were separated on a column of De-Acidite FF resin (formate form) (Ashby, Brooks & Reid, 1955).

Assays of enzymic activity. The specific assay of endopolygalacturonase in the presence of other pectolytic enzymes is difficult; a viscometric method was employed, which, although affected by exopolygalacturonase, may be expected to be much more sensitive to endopolygalacturonase (Demain & Phaff, 1957). The tendency in recent years has been to evaluate viscosity assays by determining the percentage fall in viscosity of a mixture of enzyme and pectic acid after a fixed reaction period. The percentage fall in viscosity (A) has been defined by Roboz, Barrett & Tatum (1952) as:

$$A = \frac{V_0 - V_t}{V_0 - V_s} \times 100$$

where V_0 is flow time (sec.) of pectic acid + heat-inactivated enzyme; V_t, flow time (sec.) of pectic acid + enzyme; V_s, flow time (sec.) of inactivated enzyme + solvent. Logarithmic dose–response relationships were found with assays performed in this manner (Reid, 1952; Roboz *et al.* 1952; Saito, 1955). The use of this type of assay introduced two ambiguities: first, the relationship of viscosity to average molecular size is complex; and, secondly, endopolygalacturonase has markedly different rates of attack on substrates of different sizes, so that it is not surprising that a complex relationship connects the percentage fall in viscosity with the amount of enzyme present.

These difficulties may, however, be readily avoided by determining the period taken to perform a constant amount of hydrolysis. Both Fig. 1, and the results of Roboz *et al.* (1952) and Saito (1955), show that the time required to reach a value for A of 50% is inversely proportional to the concentration of enzyme over a considerable range of activities. Throughout this work one viscosity-diminishing unit of endopolygalacturonase activity has been taken as that amount of enzyme which reduces the viscosity of 1 ml. of a 1% solution of sodium pectate by 50% in 10 min. at pH 4·8 and 30°, i.e. the number of units present in 1 ml. of the reaction mixture under the stated conditions is $10/T_{50}$, where T_{50} is the time in minutes required to reach a value for A of 50%.

It was found that 0·1 ml. graduated pipettes could be used satisfactorily as viscometers. The stem of these was

bent through an angle of about 80° at about 5 cm. from the tip, and the pipette was clamped with this 5 cm. portion hanging vertically downwards. A mouthpiece attached to the other end of the pipette with rubber tubing allowed it to be filled. In use the enzyme was diluted in 0·2 M-acetate buffer, pH 4·8, and 0·5 ml. was placed in a 3 cm. × 0·5 cm. test tube; 0·5 ml. of 2% sodium pectate was placed in a similar tube. The tubes were immersed in a water bath maintained at 30° for 2 min., and then their contents were mixed, by pouring repeatedly from one tube to the other, and the time was noted. The mixture was drawn up into the pipette at intervals, and the period taken for the upper meniscus to travel between two selected points on the scale of the pipette was noted, together with the time at which the meniscus passed the mid-point of the scale. The flow time was also determined with buffer alone and with buffer and sodium pectate. Since the enzyme preparations were usually diluted 10^4- to 10^6-fold for assay, it was not usually necessary to use heat-inactivated enzymes. A value for A was calculated for each reading obtained and a plot of this against reaction time allowed T_{50} to be determined. Before and between readings the pipette was washed out five times with 0·1 M-acetate buffer, pH 4·8, held at 30°.

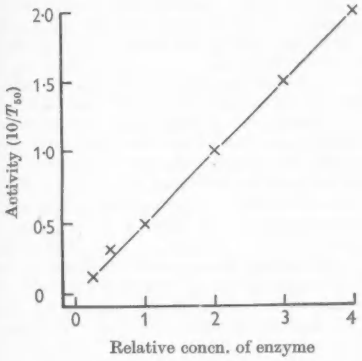

Fig. 1. Activity of various arbitrary concentrations of endopolygalacturonase measured by the time (T_{50}) required to induce a 50% fall in the viscosity of a 1% solution of pectic acid at pH 4·8 and 30°. The activities are expressed as $10/T_{50}$, with T_{50} measured in min.

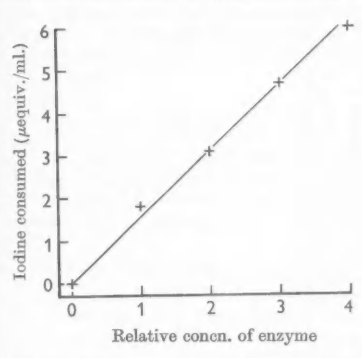

Fig. 2. Digestion of trigalacturonic acid by various arbitrary concentrations of enzyme determined by the colorimetric hypoiodite method on 0·1 ml. of a reaction mixture, containing originally 0·5% of substrate at pH 3·5, after digestion for 1 hr. at 30°.

The iodimetric method of Owens *et al.* (1952) was used in a slightly modified form to follow the release of reducing groups during pectolysis. The digestion was, as a matter of convenience, performed at 30° rather than 25° in view of the high ambient temperatures encountered in these laboratories. One unit of activity (PGu) is that which releases 1 m-mole of reducing sugar/min. from a 0·5% solution of sodium pectate at pH 4·0. Results, given as the percentage total hydrolysis, are based on the galacturonic anhydride content of the sodium pectate.

A simple colorimetric modification of the hypoiodite assay was evolved for the measurement of the release of reducing groups from purified oligogalacturonides; the method was economical of these substrates whose preparation is time-consuming. The entire reaction and assay were performed in 0·4 cm. × 10 cm. test tubes, which were selected as optically matched for use in an EEL colorimeter provided with a suitable adaptor. Replicate tubes were set up containing 0·5 ml. of a 1% neutralized solution of the substrate in 0·1 M-phthalate buffer, pH 3·5. These tubes, and suitable dilutions of the enzyme in the same buffer, were heated in a water bath at 30°, and then 0·5 ml. of enzyme was added to each tube. After a suitable period the reaction was stopped by the addition of 0·1 ml. of 4 N-Na_2CO_3; initial values were obtained by adding the Na_2CO_3 before the enzyme. After 0·5 ml. of 0·06 N-I_2 was added, each tube was stoppered. After a 20 min. reaction period, 3·4 ml. of 0·2 N-H_2SO_4 was added and the concentration of the residual I_2 was estimated in the colorimeter with filter no. 625. The colorimeter was calibrated with dilutions of standardized I_2 solution, and the assay was calibrated with galacturonic acid. The stoicheiometry of the hypoiodite assay was the same as with the normal technique (i.e. 1 m-equiv. of $I_2 \equiv 0{\cdot}51$ m-mole of sugar). Fig. 2 demonstrates the approximate linearity of the response obtained for the digestion of trigalacturonic acid by various amounts of enzyme.

Ultraviolet-absorption measurements. The extinctions of the enzyme preparations were measured at 280 mμ in a Uvispek spectrophotometer with a 1 cm. light-path. Protein concentration was taken to be proportional to extinction with $E^{1\%}_{1\,\text{cm.}} = 10$ (Dixon & Webb, 1958).

Chromatography. Chromatography of oligouronides was performed on Whatman no. 541 paper with, as solvent, the epiphase of butyric acid–acetic acid–water (200:1:200). Chromatograms were normally run for 24 hr. at 25° and the dried papers were developed by the $AgNO_3$-dip technique of Trevelyan, Procter & Harrison (1950). The series of spots produced by the chromatography of a partially hydrolysed sample of pectic acid gave a straight line when the logarithms of the distances which they travelled were plotted against their presumed molecular sizes, and the mono-, di- and tri-mer were identified by comparison with authentic samples of these purified uronides.

Carboxymethylcellulose. This was prepared as described by Peterson & Sober (1956), and contained 0·5 m-equiv./g.

Buffer solutions. These were formulated according to Gomori (1955), from analytical-grade reagents.

Paper electrophoresis. This was performed on a horizontally supported Whatman no. 1 paper, at 10–20 v/cm., for 20 hr. The protein bands were revealed with bromophenol blue in alcoholic $HgCl_2$ solution (Kunkel & Tiselius, 1951).

pH *measurements.* Where possible these were made with a glass electrode. However, when very small volumes were involved, as in the latter stages of the purification, Special Indicator Papers (E. Merck A.G., Darmstadt, Germany) were used.

Purification of endopolygalacturonase

Fermentation. This was continued for 5 days; Fig. 3 shows the titre of endopolygalacturonase activity, in viscosity-diminishing units, reached at various times during the fermentation. At the end of this period the culture fluid was filtered under vacuum and held at 4° until required.

The following purification scheme describes the quantities of reagents used for 10 l. of culture fluid.

Stage 1. Small portions of the culture fluid were added to various amounts of 0·1 N-$NaHCO_3$, and the pH values of the resultant mixtures were measured with the glass electrode. A titration curve, constructed from the results, was used to calculate the volume of 0·1 N-$NaHCO_3$ required to adjust the bulk of the culture fluid to pH 4·0. The solution was added slowly to the fluid, cooled to 0–2° and stirred mechanically. Cold 95% ethanol (2·5 l.) was added and after 1 hr. the small precipitate was removed. The slow addition of 16·1 l. of cold ethanol raised its concentration to about 65%, and, after the mixture had been kept at −8° to −10° for 2 hr., the precipitated enzyme was harvested by centrifuging.

Stage 2. The precipitate was suspended in 500 ml. of saturated $(NH_4)_2SO_4$ solution at 2°, the resultant volume was measured and the volume of the precipitate estimated by subtracting the 500 ml. volume of $(NH_4)_2SO_4$. For each 100 ml. of precipitate, 50 g. of solid $(NH_4)_2SO_4$ was added. The whole was thoroughly dispersed in a Waring Blendor for 20 min., and then 10 g. of Celite was added and the mixture filtered under vacuum; the residue was resuspended in 125 ml. of 95% saturated $(NH_4)_2SO_4$, stirred for 1 hr. and again filtered through Celite.

The filter cake was resuspended in 250 ml. of 50% saturated $(NH_4)_2SO_4$ and the mixture stirred for 1 hr. and then filtered as before; the residue was once more suspended in 125 ml. of 50% saturated $(NH_4)_2SO_4$, stirred for 1 hr. and filtered.

The combined 50% saturated $(NH_4)_2SO_4$ filtrates were dialysed for 24 hr. against running tap water in a rocking dialyser. A double thickness of Visking cellophan tubing was used as the dialysis membrane, to avoid rupture due to the cellulase present in the preparation.

The material at this stage was reasonably stable and could be stored in solution at 4° for 2–3 weeks.

Stage 3. The last traces of salt were removed from the solution of the enzyme by passing it through a mixed resin column of 40 ml. of Amberlite IR-120 (H^+) and 76 ml. of Amberlite IR-400 (OH^-). The column was washed with 500 ml. of water, which was then added to the eluate. The pH of the mixture was adjusted to 4·2–4·4 by the addition of 0·05 N-acetic acid. Carboxymethylcellulose (6 g.) was suspended in N-acetic acid, filtered off and washed on the filter with three 100 ml. portions of 0·05 M-acetate buffer, pH 4·4. The carboxymethylcellulose was added to the enzyme solution, the mixture was stirred for 30 min. at 4° and then filtered. The residue was washed with two 50 ml. portions of 0·05 M-acetate buffer, pH 4·4, and then suspended in 36 ml. of 0·05 M-acetate buffer, pH 4·6; the pH of the mixture was readjusted to 4·6 by the cautious addition of 0·05 N-sodium acetate. The mixture was stirred for 30 min. and filtered, and the residue washed on the filter with two 36 ml. volumes of 0·05 M-acetate buffer, pH 4·6.

The carboxymethylcellulose was resuspended in 36 ml. of 0·05 M-acetate buffer, pH 5·3, and the pH was adjusted to 5·3 with 0·05 M-sodium acetate. After stirring for 30 min. the mixture was filtered; the carboxymethylcellulose was resuspended in 18 ml. of 0·05 M-acetate buffer, pH 5·3, stirred for 10 min. and filtered, and the filtrate combined with the previous one.

Stage 4. The combined filtrates were treated with small portions of Amberlite resin IR-120 (H^+) until a pH of 4·2–4·4 was obtained. The solution was decanted from the resin, which was washed twice with a little distilled water, the washings being added to the rest of the solution. One gram of carboxymethylcellulose, adjusted to pH 4·4 as before, was added and the mixture stirred for 30 min. The carboxymethylcellulose was filtered off, washed on the filter with two 5 ml. portions of 0·05 M-acetate buffer, pH 4·4, and resuspended in 6 ml. of 0·05 M-acetate buffer, pH 4·6. The mixture was adjusted to pH 4·6 with 0·05 N-sodium acetate, stirred for 30 min. and filtered, and the residue washed on the filter with two 6 ml. portions of 0·05 M-acetate buffer, pH 4·6.

The carboxymethylcellulose was resuspended in 6 ml. of 0·1 M-acetate buffer, pH 5·0, and the mixture was stirred for 30 min. and filtered. The carboxymethylcellulose was again suspended in 3 ml. of 0·1 M-acetate buffer, pH 5·0; the mixture was stirred for 30 min. and filtered, and the filtrate combined with the previous one. The pH of the combined solution was cautiously adjusted to 6·0 with 0·2 M-sodium acetate. This solution constituted the partially purified enzyme preparation used for the experiments described below. Table 1 summarizes the progress of the

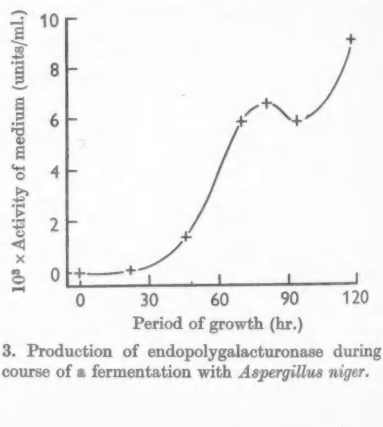

Fig. 3. Production of endopolygalacturonase during the course of a fermentation with *Aspergillus niger*.

Table 1. *Purification of endopolygalacturonase from 6 l. of culture fluid*

Fraction	10^{-3} × Specific activity (units/$E^{1cm.}_{280 m\mu}$)	Relative activity	10^{-6} × Total activity (units)	Percentage of original activity
Culture fluid	0·61	1	44	100
Stage 1. Fraction pptd. between 20 and 65% (v/v) of ethanol at pH 4·0	2·9	5	35	80
Stage 2. Fraction insoluble in 95% but soluble in 50% saturated $(NH_4)_2SO_4$	15	24	33	76
Stage 3. Fraction eluted from carboxymethylcellulose by 0·05 M-acetate, pH 4·6–5·3	65	100	27	61
Stage 4. Fraction eluted from carboxymethylcellulose by acetate between 0·05 M, pH 4·6, and 0·1 M, pH 5·0	280	400	15	34

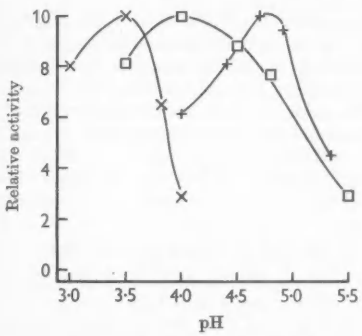

Fig. 4. pH optima for the activity of endopolygalacturonase measured in terms of (*a*) the diminution in viscosity of a 1% solution of pectic acid (+), (*b*) the release of reducing groups from a 0·5% solution of pectic acid (□), (*c*) the release of reducing groups from a 0·5% solution of trigalacturonic acid (×).

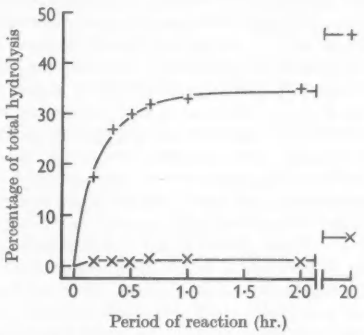

Fig. 5. Hydrolysis of 1% solutions of pectin (×) and pectic acid (+), measured by the release of reducing groups at pH 4·0.

purification. The final preparation contained about 35 mg. of protein on an extinction basis with a specific activity of some $8·8 \times 10^5$ viscosity-diminishing units/mg. of protein. Numerous preparations were performed over a period of a year without failure.

RESULTS

Paper electrophoresis. In phosphate buffers of pH 7·2 and 8·0, and ionic strength 0·15, the partially purified preparation gave a single sharply defined band on paper electrophoresis.

Action of the enzyme against pectin and pectic acid

Reduction of viscosity. The enzyme produced a rapid fall in the viscosity of solutions of pectin and pectic acid at pH 4·8. However, the preparation was 2·7 times as active, in this respect, when acting on pectic acid as on pectin.

To determine the optimum pH for the action of the enzyme against pectic acid, 0·5 ml. portions of 2% sodium pectate at the desired pH values were mixed with 0·5 ml. of dilutions of the enzyme in 0·2 M-acetate buffers of the same pH values and the fall in viscosity followed in the normal manner; the pH values of the digests were then measured. The enzyme showed its maximum activity at pH 4·7–4·8 (Fig. 4*a*).

Release of reducing groups. There were marked differences from the previous results when the activity of the enzyme was followed by measuring the release of reducing groups; pectic acid rapidly undergoes a 30–40% hydrolysis but pectin is little affected (Fig. 5). The optimum pH for the release of reducing groups from pectic acid was determined by mixing 4 ml. of 1% sodium pectate, preadjusted to the desired pH, 3 ml. of 0·2 M-acetate buffer, at the same pH, and 1 ml. of a suitable dilution of the enzyme in water. After incubation for 10 min. at 30°, the reaction was stopped by adding 2 ml. of 2 N-Na_2CO_3, and the reducing groups were determined by the hypoiodite method. Starting blanks were obtained by adding the ingredients to flasks containing the 2 N-Na_2CO_3. An optimum for the reaction was obtained at pH 4·0–4·2 (Fig. 4*b*). Enzyme concentrations were adjusted so that all

determinations were made within the initial region of rapid hydrolysis.

The breakdown of pectic acid was very rapid initially but became very slow after 40 % hydrolysis has been obtained; nevertheless, in the presence of very high enzyme concentrations it will continue to at least 60 %. With a digestion mixture containing 0·5 % of pectic acid and 6000 viscosity-diminishing units of enzyme/ml., the level of digestion had reached 35 % after 2 min. and 41, 54 and 61 % after 44, 145 and 185 hr. respectively.

The initial stages of pectic acid hydrolysis were followed by spotting on chromatographic paper 0·01 ml. portions of a reaction mixture of 0·5 % sodium pectate and 60 viscosity units of enzyme/ml., after various short periods of reaction. The spots were dried immediately in a stream of air, and then the paper was developed in the butyric acid–acetic acid–water solvent. The sample taken after digestion for 2 min. showed faint spots corresponding to mono-, tri-, tetra-, penta- and hexa-galacturonic acid; these were joined by the digalacturonic acid spot in the 4 min. sample. The intensities of all the spots now increased with increasing reaction time up to the final sample at 30 min. When higher enzyme concentrations and longer reaction times were used in order to make a similar examination of the later stages of the reaction, the spots corresponding to the higher galacturonides progressively disappeared with increasing reaction time. The disappearance of the trigalacturonic acid spot was very slow and the digalacturonic acid spot remained.

Action of the enzyme against purified oligogalacturonides

The action of the enzyme against tetra-, tri- and di-galacturonic acid was followed chromatographically by mixing 0·5 % solutions of the neutralized acids with suitable concentrations of enzyme and spotting samples of the mixture on paper as described for the pectic acid. Tetragalacturonic acid underwent a fairly rapid split to tri- and mono-galacturonic acid, and trigalacturonic acid underwent a much slower split to di- and mono-galacturonic acid. Digalacturonic acid, however, was not attacked.

The actual rate of breakdown of trigalacturonic acid was measured by the colorimetric hypoiodite method, and it was confirmed that the digalacturonic acid was resistant to hydrolysis by this enzyme; Table 2 compares the rates of hydrolysis of pectic acid (at pH 4·0) and tri- and di-galacturonic acid (at pH 3·5) under the influence of the enzyme preparations stage 1 and stage 4 used at equivalent viscosity-diminishing activities.

The optimum pH for the hydrolysis of trigalacturonic acid by the enzyme preparation stage 4 was found by measuring the rate of hydrolysis of this substrate by the colorimetric hypoiodite method, with, as reaction mixtures, 0·4 ml. of each of a suitable range of 0·2 M-buffers, 0·1 ml. of a suitable dilution of the enzyme in water and 0·5 ml. of a neutralized 1 % solution of trigalacturonic acid. Maximum activity was at pH 3·5 (Fig. 4*c*).

Stability of endopolygalacturonase

The effect of pH on stability of the enzyme at 50° and 80° was investigated by diluting a solution of the enzyme to 1000 viscosity-diminishing units/ml. with each of a range of 0·1 M-citrate–phosphate buffers. A portion (3 ml.) of each dilution, the pH of which was checked, was then placed in stoppered tubes and heated for exactly 10 min. in a large water bath held at the desired temperature. The tubes were quickly cooled and a small portion of

Table 2. *Relative rates of hydrolysis of different substrates by pectic enzyme preparations*

Figures are given for the rates of hydrolysis of the substrates shown with enzyme preparations stage 1 and stage 4. Hydrolyses were performed at 30° with 0·5 % substrate concentration throughout.

Substrate	pH	PGu/10⁶ viscosity-diminishing units: Stage 1	Stage 4
Pectic acid	4·0	10·3	8·0
Trigalacturonic acid	3·5	0·065	0·009
Digalacturonic acid	3·5	0·022	0·00

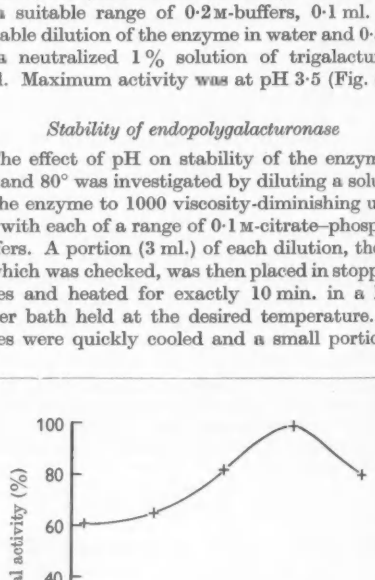

Fig. 6. Effects of pH and temperature on the stability of endopolygalacturonase in a solution containing 1000 viscosity-diminishing units/ml. Samples at the various pH values shown were held at 50° (+), and at 80° (×), for 10 min.

their contents was withdrawn and immediately diluted with 0·2 M-acetate buffer, pH 4·8, before assay by the viscosity method. A narrow range of enhanced stability was found about pH 6 (Fig. 6).

When stored at 5° and pH 6 the preparation stage 4 lost some 15 % of its activity in 5 days. Attempts to increase the stability by the addition of pectin, sucrose, galacturonic acid, gelatin, BAL (2:3-dimercaptopropanol), cysteine or ethylenediaminetetra-acetic acid were uniformly unsuccessful. The preparation lost much of its activity when air was bubbled through it or when it was frozen and thawed.

DISCUSSION

The method of purification described here has resulted in an approximately 400-fold purification of the endopolygalacturonase from culture fluids of *A. niger*. The only other clear investigation of this enzyme from this source which could be found in the literature is that of Saito (1955). This author selected a unit of viscosity-diminishing activity such that 10 units would diminish the viscosity of 5 ml. of a 0·8 % solution of pectic acid by 50 % in 1 hr. at pH 4·2 and 30°. It is especially difficult to compare the results of viscosity assays by authors using different types of pectic acids (Saito used a citrus pectic acid with a uronic anhydride content of only 69 %), but so far as such a comparison is valid it would appear that one of our units of activity is equivalent to about 12 of Saito's. His purified material had an activity of 3000 of his units per mg. (equivalent to 250 of our units per mg.). Our preparations are thus more than 1000-fold as active as those of Saito.

Lineweaver, Jang & Jansen (1949) purified a polygalacturonase of unspecified type from the commercial preparation Pectinol, obtaining an activity of 1·41 PGu/mg. of total nitrogen. This suggests a value of 0·23 PGu/mg. of protein. Our preparations have activities of more than 2 PGu/mg. of protein.

The pattern of production of oligogalacturonides during the hydrolysis of pectic acid by the enzyme shows that there is a random mechanism of attack of this substrate which, taken with the high activity found in the viscosity assay, clearly demonstrates that an 'endo' enzyme is involved. The enzyme will rapidly reduce the viscosity of a pectin solution but the activity is slower than that with pectic acid. Reid (1952) calculated that the scission of only 0·5–1 % of the internal glycosidic bonds of pectic substances would reduce the viscosity of their solution by 50 %. Since very nearly half of the carboxylic groups in our pectin remain unesterified it is not surprising that some length of the polymer should be susceptible to an endopolygalacturonase, i.e. to an enzyme which preferentially attacks the bond linking two unesterified galacturonic acid residues. Moreover, the very slight release of reducing groups which the enzyme brings about from pectin makes it clear that an endopolygalacturonase is indeed involved.

The fall in the noted optimum pH values which we found in the viscosity assay, the release of reducing groups from pectic acid and the release of the reducing groups from trigalacturonic acid are consistent with the observation of Demain & Phaff (1954) and Saito (1955) that the smaller the substrate size the lower was the optimum pH; in the three assays mentioned the mean substrates are respectively pectic acid, high-molecular-weight oligouronides produced by 5–10 % hydrolysis of pectic acid, and the low-molecular-weight trigalacturonic acid. The values of 4·7–4·8 for the viscosity assay and 4·0–4·2 for the reducing-group assay are in good agreement with those reported by Saito.

The question of the range of substrate sizes attacked by endopolygalacturonase is particularly difficult. The endopolygalacturonase of the yeast *S. fragilis* will attack all of the galacturonides except digalacturonic acid (Demain & Phaff, 1954; Phaff & Demain, 1956; Patel & Phaff, 1959). Brooks & Reid (1955) found that a similar enzyme from *Aspergillus foetidis* could not attack either di- or tri-galacturonic acid. Saito's (1955) enzyme, also, did not apparently attack these substances. But McCready & Seegmiller (1954) reported that their enzyme from Pectinol attacked both these uronides at a rate about 5 % of that at which it attacked pectic acid.

Our preparation stage 4 induces a slow but definite hydrolysis of trigalacturonic acid but is completely devoid of action against digalacturonic acid. However, one single preparation of stage 4 did produce a slight hydrolysis of digalacturonic acid; this result could not be repeated with other batches and was doubtless due to the presence of a contaminating enzyme in the preparation. That preparation stage 1 contains another pectic enzyme is clear, in that this preparation splits digalacturonic acid at an appreciable rate and trigalacturonic acid at a much higher rate than does preparation stage 4.

The behaviour of *A. niger* endopolygalacturonase is thus analogous with that of *S. fragilis*; Demain & Phaff (1954) postulated that one of the terminal residues of the galacturonide chain protected the adjacent glycosidic bond completely, and the next glycosodic bond partially, against attack by yeast endopolygalacturonase. Apart from this proviso all the other bonds of the chain were supposed to be freely susceptible to attack. This hypothesis satisfactorily explains the action of the *A. niger* enzyme also.

SUMMARY

1. Endopolygalacturonase has been separated from the culture fluid of *Aspergillus niger* with a purification factor of 400-fold and a recovery of 34%.

2. The preparation obtained was homogeneous on paper electrophoresis at pH 7·2 and pH 8·0, and of moderate stability with an optimum for stability of about pH 6.

3. The enzyme rapidly lowered the viscosity of a solution of pectic acid and released reducing groups from it according to a random pattern of hydrolysis, but the digestion was not complete. Pectin underwent only a very slight degradation, sufficient to show a fall in viscosity but with little release of reducing groups.

4. Tetragalacturonic acid was rapidly split to tri- and mono-galacturonic acid, and trigalacturonic acid was slowly split to di- and mono-galacturonic acid, but the digalacturonic acid was not attacked.

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Chemical Reactions which Affect the Biological Activity of Human Gonadotrophins

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The biological activity of the gonadotrophic hormones is diminished by the action of various reagents. The effects of oxidation, reduction, acetylation and enzymic hydrolysis have been extensively investigated and certain differences in the relative stabilities of follicle-stimulating hormone (FSH) and interstitial-cell-stimulating hormone (ICSH) have been reported.

The gonadotrophins are glycoproteins and the carbohydrate groups appear to be important for biological activity. Thus Whitten (1950) demonstrated that the activity of ICSH in serum gonadotrophin was retained after treatment with 2 mM-periodate but the activity of FSH was diminished. Geschwind & Li (1958), however, claimed that both gonadotrophins prepared from the anterior-pituitary glands of sheep were destroyed by treatment with 0·2 M-potassium periodate.

Fraenkel-Conrat, Simpson & Evans (1939, 1940) and McShan & Meyer (1940) showed that reduction

with cysteine diminished the activity of sheep FSH, and Li, Simpson & Evans (1940) and Fraenkel-Conrat *et al.* (1940) showed that it completely destroyed sheep ICSH. Similar results were obtained after acetylation with keten (Li, Simpson & Evans, 1939). The activity of ICSH may therefore be connected with the presence of –S–S– linkages reducible by cysteine or with amino groups accessible to keten.

Other differences between FSH and ICSH have been demonstrated by experiments with enzymes. Chow, Greep & van Dyke (1939) found that under certain conditions hog FSH is stable to tryptic digestion and is only partially destroyed by chymotrypsin, but that hog ICSH is completely destroyed by both.

Reactions with certain cations have also been studied. Antoniades *et al.* (1957) used precipitation with zinc and ethanol for the extraction of gonadotrophin from human plasma, and Legault-Démare, Clauser & Jutisz (1958) claimed that a 20-fold increase in the purity of pregnant-mare serum gonadotrophin was obtained by the removal of inert material precipitable by barium.

These observations on gonadotrophins extracted from the anterior pituitary gland and from the serum of various animals led us to investigate similar reactions with the human gonadotrophins. The study was designed to show whether any reaction differentially affected the biological activity of FSH or ICSH. Such a reaction might be of practical value in the purification of the hormones from human urine or pituitary tissue.

METHODS

Biological assay

Immature female mice of an albino strain (supplied by Mr W. Clarke, Oldham), weighing 8–10 g. at the start of the assay, were used in each test. The gonadotrophin in 0·9% sodium chloride soln. was injected subcutaneously in five equal doses of 0·5 ml. over a 3-day period. Injections were made twice a day on the first 2 days and once on the third. The animals were killed 72 hr. after the first injections. The uteri or ovaries were dissected and weighed fresh.

Three to six animals were used for each dose and the interval between successive doses was either 1·5 or 2·0. Two methods were used, which have been described in detail by P. S. Brown (1955).

(*a*) Uterine-weight method. This is believed to measure the combined effect of follicle-stimulating hormone (FSH) and interstitial-cell-stimulating hormone (ICSH). For the construction of the dose–response curve the logarithm of the dose and the mean of the combined uterine weights were used. The response was linear with doses between 0·5 and 2·0 mg. of the International Reference Preparation (HMG 24). Whenever possible at least three doses were used so that the linearity of the response line could be checked.

(*b*) Augmentation method. This is believed to be relatively specific for FSH. It depends on the increase in weight of the ovary of the immature mouse treated simultaneously with the test material and with a great excess (40 i.u.) of human chorionic gonadotrophin (HCG). The HCG has an action similar to ICSH and causes only a small increase in ovarian weight. The effect of ICSH in the test material is rendered insignificant by the excess of HCG. Any increase in weight of the ovary above that caused by the HCG alone is considered to be due to FSH. The specificity of this method has been discussed elsewhere (Brown, 1959). The log dose–response curve was linear in the range 0·5–1·5 mg. of HMG 24.

The results were calculated by the standard methods given by Bliss (1952) or by the simplified methods of Gaddum (1953). The activity of the material recoverable after chemical treatment was calculated from the assay results and expressed as a percentage of the activity in the original material. The results of two assays are considered to be qualitatively similar if their 95% fiducial limits of error overlap and the ratio of calculated potencies (index of discrimination) by the two methods does not exceed two (Cunningham & Brown, 1958).

A laboratory substandard was prepared from urine of post-menopausal women by the benzoic acid–tungstic acid method of Butt (1958). Its potency was determined by both assays in terms of HMG 24: 1 mg. was equivalent to 0·73 (95% fiducial limits 0·62–0·84) mg. of HMG 24 by the uterine-weight method and 1·04 (0·37–2·10) mg. of HMG 24 by the augmentation method.

Preparation of gonadotrophin for chemical experiments

Urine. Urine of post-menopausal women was extracted by the benzoic acid–tungstic acid method of Butt (1958) or by the kaolin-adsorption method (Brown, 1959). The powders obtained were then purified by use of ion-exchange materials (Butt & Round, 1958). The mixed resin, Bio-Deminrolit (The Permutit Co.), was used in the first stage of this procedure instead of De-Acidite FF in the borate form, as previously described, since less preparation of the resin was required and equally good results were obtained. The active material was then adsorbed on diethylaminoethyl (DEAE)-cellulose prepared from Whatman cellulose powder (Peterson & Sober, 1956) and was eluted by 0·5M-ammonium acetate (at pH 9·5) made up in 0·2M-sodium chloride as previously described. The potency of different batches measured by the two methods of assay varied slightly but was usually similar to that of the International Reference Preparation (HMG 24). These products therefore contained both FSH and ICSH in about the same proportions as the standard.

Pituitary gland. A powder was supplied by Dr A. Korner (University of Cambridge, Department of Biochemistry) which had been prepared from acetone-dried human pituitaries as a by-product in the extraction of growth hormone. This preparation involved extraction of the acetone-dried powder in potassium chloride (0·34M) at pH 5·5, after which the pH was adjusted to 8·6. Ethanol was added until the concentration was 30% (v/v) and any precipitate was removed by centrifuging. Solid ammonium sulphate was added to 50% saturation (based on the water content) and the precipitate was discarded. The super-

natant was dialysed and freeze-dried and the dry powder was then fractionated in this laboratory.

It was first applied to a column of carboxymethyl (CM)-cellulose as described by Steelman, Segaloff & Andersen (1959) but ammonium acetate was used instead of sodium acetate for elution. The fraction eluted in 0·01 M-ammonium acetate was precipitated by adding 3 vol. of ethanol and then was dissolved in water and fractionated on DEAE-cellulose as described by Butt, Crooke & Cunningham (1959) for urinary gonadotrophin. The material eluted from DEAE-cellulose in 0·2 M-ammonium acetate was precipitated by adding 3 vol. of ethanol, washed with ethanol and ether and dried. Biological assay by the uterine-weight method showed that 1 mg. was equivalent to 86 (65–144) mg., and by the augmentation method to 240 (180–320) mg., of the standard HMG 24. Histological examination of the ovaries of hypophysectomized rats given this material showed that it was a potent follicle-stimulating hormone and it will be referred to in this paper as pituitary 'FSH'. Ultracentrifugal analysis by Dr D. R. Stanworth (University of Birmingham, Department of Experimental Pathology) indicated that it contained one major component with a sedimentation coefficient, $S_{20,w}$, 3·95. Centrifuging was performed in barbitone buffer (pH 8·6; I 0·05; 0·2 M-sodium chloride) at 60 000 rev./min. at 20°. After sedimentation for 144 min. there was a suggestion of some heterogeneity in the form of a slight shoulder on the trailing edge.

Material eluted in M-ammonium acetate from the CM-cellulose column was reprecipitated by adding 3 vol. of ethanol and then refractionated on CM-cellulose. The eluate in 0·1 M-ammonium acetate was precipitated by adding 3 vol. of ethanol, washed with ethanol and ether and dried. There was insufficient material for ultracentrifugal analysis. The product from approx. 20 mg. of material was dissolved in 1 ml. of 0·9% sodium chloride soln. It contained the equivalent of 780 (480–1460) mg. of HMG 24 by the uterine-weight method and 850 (255–1720) mg. of HMG 24 by the augmentation method. This shows that it is qualitatively similar to the standard by these assays but it differs from the standard in its effect on the ovaries of immature mice in that corpora haemorrhagica are produced by a dosage of only twice that which is required to double the uterine weight. It corresponds with the luteinizing or interstitial-cell-stimulating hormone fractions of Steelman *et al.* (1959) and for this reason it will be referred to as pituitary 'ICSH'.

EXPERIMENTAL AND RESULTS

In all experiments, unless otherwise stated, the products were finally precipitated by adding 5 vol. of ethanol and were washed with ethanol and ether.

Oxidation

Periodate. Gonadotrophin (20 mg. of urinary, or 200 μg. of pituitary FSH) in 2 ml. of 0·2 M-ammonium acetate at pH 7·0 was mixed with 2 ml. of 4 mM-potassium periodate. The reaction proceeded for 30 min. at room temperature and was then stopped by the addition of excess of glucose.

Hydrogen peroxide. Urinary gonadotrophin (20 mg.) was dissolved in water (4 ml.) and 0·25 ml. of 6% (w/v) hydrogen peroxide was added. Two further portions of 0·25 ml. of hydrogen peroxide were added at 15 min. intervals. Oxidation proceeded for a further 45 min. and the gonadotrophin was then reprecipitated.

The potency of FSH and ICSH was partially diminished by the effect of periodate and was considerably diminished by hydrogen peroxide but there was no significant difference between the results by the two methods of assay (Table 1).

Reduction

Urinary gonadotrophin (20 mg.) was dissolved in 1 ml. of 0·1 M-ammonium acetate (pH 7·0) and was reduced at room temperature by: (*a*) ascorbic acid (20 mg.) for 6 hr.; (*b*) thioglycollic acid (0·2ml.) for 6 hr.; (*c*) methionine (20 mg.) for 6 hr.; (*d*) cysteine (20 mg.) for 6 hr.; (*e*) sodium borohydride (8–20 mg.) for 1 hr.

There was slight loss of active material after treatment with ascorbic acid, thioglycollic acid or methionine and rather more loss with cysteine (Table 2). Reduction with sodium borohydride (0·4 mg./mg. of protein) caused no loss of activity but with 1·0 mg./mg. of protein the activity was modified. Results by the uterine-weight method showed that less than 33% of the original activity remained whereas in the augmentation assay for

Table 1. *Oxidation of human urinary gonadotrophins*

The 95% fiducial limits are given in parentheses. All assays were of at least 2:2 point design except in the experiment with H_2O_2, which was of 1:3 point design. Divergence from parallelism of the dose–response lines is not significant (P 0·05) in any assay. The index of precision (λ) is calculated by dividing the standard deviation of the responses by the slope of the line connecting the response with the logarithm of the dose. It varied in these assays between 0·05 and 0·23.

Method of oxidation	Type of gonadotrophin	Relative potency (% of control)	
		Augmentation assay for FSH	Uterine-weight assay for FSH + ICSH
2 mM-Periodate	Urine	55 (38–68)	38 (31–47)
	Pituitary 'FSH'	35 (22–47)	46 (36–57)
	Pituitary 'ICSH'	30 (19–62)	36 (4–65)
H_2O_2	Urine	$\ll$40	$\ll$30

Table 2. *Reduction of human urinary gonadotrophin*

The designs of the assays are shown by the number of doses of reduced (first number) and initial materials (second number) and the significance of the divergence in slopes of the dose–response lines [$P(G)$] is also given. The index of precision (λ) in these assays varied between 0·06 and 0·17.

Method of reduction	Design	Augmentation assay for FSH (relative potency as % of control with 95% fiducial limits)	$P(G)$	Design	Uterine-weight assay for FSH + ICSH (relative potency as % of control with 95% fiducial limits)	$P(G)$
Ascorbic acid	2:2	71 (19–76)	0·6–0·7	2:3	51 (36–84)	0·9
Thioglycollic acid	2:3	73 (59–77)	0·6–0·7	2:3	53 (30–77)	0·9
Methionine	1:3	78 (55–110)	—	1:3	140 (80–1900)	—
Cysteine	1:2	51 (20–68)	—	1:3	43 (5–74)	—
Sodium borohydride						
0·4 mg./mg.; 1 hr.	2:3	150 (85–447)	0·6–0·7	3:3	81 (36–168)	0·1–0·2
1·0 mg./mg.; 1 hr.	3:3	Invalid due to divergence in slopes of dose–response lines	0·001	1:3	<33	—

Table 3. *Treatment of urinary and pituitary gonadotrophin with borohydride*

All the assays were of at least 2:2 point design except in the experiments with pituitary 'ICSH', where the design was 1:2. There was no significant deviation from parallelism in any assay and the index of precision (λ) varied between 0·07 and 0·20.

Preparation	Potency (% of control with 95% fiducial limits): Augmentation assay for FSH	Potency (% of control with 95% fiducial limits): Uterine-weight assay for FSH + ICSH
Urinary 1	72 (68–78)	34 (30–43)
Urinary 2	93 (56–215)	36 (16–59)
Urinary 3	87 (51–132)	49 (40–70)
Pituitary 'FSH'	67 (50–104)	66 (28–142)
Pituitary 'ICSH'	<12	<20

Table 4. *Effect of sodium hydroxide on activity of gonadotrophins*

Gonadotrophins were prepared by the benzoic acid–tungstic acid method. They were dissolved in water and brought to pH 11·5 with N-NaOH. After 20 min. the pH was readjusted to approx. 7·0 and the gonadotrophin was reprecipitated by adding 5 vol. of ethanol. Assays were of 2:2 point design and the index of precision (λ) varied between 0·05 and 0·10.

	Potency (% of control with 95% fiducial limits): Augmentation assay for FSH	Potency (% of control with 95% fiducial limits): Uterine-weight assay for FSH + ICSH
Post-menopausal gonadotrophin	123 (101–150)	96 (80–137)
Male gonadotrophin	—	95 (76–115)

FSH the slope of the log dose–response line was significantly less than the original.

Reduction with borohydride was therefore re-investigated. Three different samples of urinary gonadotrophin and samples of pituitary 'FSH' and 'ICSH' were reduced with 1·0 mg. of sodium borohydride/mg. of gonadotrophin for 1 hr. The potencies of the products were then compared by bioassay with the respective controls (Table 3). In the experiments with urinary gonadotrophin the results of the uterine-weight assays were always lower than those of the augmentation assays. This suggests that ICSH is destroyed by borohydride more readily than is FSH. The experiments with pituitary 'FSH' and 'ICSH' support this conclusion since the potency of FSH is only slightly diminished whereas that of ICSH is greatly diminished. In contrast with the previous experiment in Table 2, there was no evidence of lack of parallelism in the log dose–response lines in these assays.

Effect of alkali

Gonadotrophin prepared by the benzoic acid–tungstic acid method was dissolved in water and brought to pH 11·5 with N-sodium hydroxide (glass electrode). After 20 min. at 20° the pH was adjusted to approx. 7·0 and the gonadotrophin was reprecipitated.

This treatment had no effect on the potency of gonadotrophin from the urine of post-menopausal women or from normal men (Table 4).

Other reactions

Methylation. About 150 mg. of urinary gonadotrophin was dissolved in 22 ml. of water, which was then divided equally in experimental and control

tubes. To each tube was added 0·18 g. of boric acid and 0·8 ml. of 20% sodium hydroxide. After shaking to dissolve the boric acid, 0·2 ml. of dimethyl sulphate was added to the experimental tube and 0·2 ml. of water to the control. The tubes were shaken and left at room temperature for 15 min., when a further 0·2 ml. of dimethyl sulphate and 0·4 ml. of 5N-sodium hydroxide were added to the experimental tube and 0·6 ml. of water to the control, and left for 15 min. About 2 g. of Bio-Deminrolit was then added to each tube and after filtration the gonadotrophins were precipitated by adding 4 vol. of ethanol and were washed with ethanol and ether.

The potency of the methylated gonadotrophin was less than 19% of the initial potency by both methods of assay. Methoxyl groups were measured by the Zeisel method. The control preparation contained no detectable methoxyl groups whereas the methylated preparation contained 1·8%.

Hydroxylamine. Urinary gonadotrophin (about 80 mg.) was dissolved in 10 ml. of 0·2M-ammonium acetate (pH 7·4) and divided equally between experimental and control tubes. Hydroxylamine hydrochloride (2 ml.) was brought to pH 7·4 with sodium hydroxide and added to the experimental tube and 2 ml. of water added to the control. After 1 hr. at 15° no precipitate had formed in the experimental tube and the gonadotrophins were therefore precipitated with 3 vol. of ethanol. The precipitates were washed five times with ethanol and finally with ether. There was no loss of FSH or ICSH by this treatment (Table 5).

Treatment with salivary amylase. Urinary gonadotrophin (about 10 mg.) was dissolved in 6·0 ml. of 0·5M-ammonium acetate (pH 6·6) and divided equally between experimental and control tubes. Filtered saliva (6 ml.) was added to the experimental tube and 6 ml. of water to the control. After 4 hr. at 25° each was treated with 4 g. of Bio-Deminrolit for 10 min. This amount was sufficient to remove the amylase present. After filtration the gonadotrophins were precipitated by adding 3 vol. of ethanol in the cold, and washed with ethanol and ether. This treatment did not decrease the potency of FSH or ICSH (Table 5).

Combination with cations

Zinc. The method of Antoniades *et al.* (1957) was used in this experiment. A portion (0·02 ml.) of a mixture of 0·25M-zinc acetate and 0·25M-glycine was added to urinary gonadotrophin (20 mg.) in 1 ml. of water. The mixture was refrigerated (5°) for 30 min. and the precipitate separated by centrifuging. It was washed with a mixture of 1 ml. of water and 0·02 ml. of zinc reagent and the wash was combined with the first supernatant. A further 0·5 ml. of the zinc reagent and ethanol (0·6 ml.) was then added to the combined washes and the precipitate which again formed was separated by centrifuging. A further 7·5 ml. of ethanol was added to the supernatant and a third precipitate was separated by centrifuging. The three precipitates were each taken up separately in small volumes of 0·15M-sodium chloride and 0·1M-sodium citrate to dissolve the zinc. The protein in each was then precipitated by the addition of 5 vol. of ethanol and the precipitates were finally washed with ethanol and ether. All the detectable biological activity was recovered in the second precipitate (Table 6) and there was no significant difference between results by the two assays. The recovery of active material was good.

Copper. A portion (0·2 ml.) of 0·2M-cupric sulphate was added to urinary gonadotrophin (20 mg.) in 1 ml. of 0·2M-ammonium acetate. The precipitate which formed was separated by centrifuging and dissolved in 0·2M-ethylenediaminetetra-acetic acid (sodium salt). Protein was reprecipitated by adding 5 vol. of ethanol and was washed with ethanol and ether. The recovery of biologically active material in this fraction is given

Table 5. *Treatment of urinary gonadotrophin with salivary amylase and with hydroxylamine*

Gonadotrophins were prepared by the kaolin method. The 95% fiducial limits are given in parentheses. The design in each assay was 2:2. The divergence from parallelism in the dose–response lines was not significant and the index of precision (λ) varied between 0·08 and 0·14.

	Potency (% of control with 95% fiducial limits)	
	Augmentation assay for FSH	Uterine-weight assay for FSH + ICSH
Hydroxylamine	100 (78–158)	125 (101–157)
Amylase	135 (68–168)	159 (112–250)

Table 6. *Precipitation of gonadotrophins with zinc, copper and barium*

In experiments with zinc and copper the gonadotrophin was precipitated by the metal: in the experiment with barium the gonadotrophin remained in solution. There was no evidence of any divergence from parallelism in the dose–response lines in these assays and the index of precision (λ) was between 0·05 and 0·26.

	Potency (% of control with 95% fiducial limits)	
	Augmentation assay for FSH	Uterine-weight assay for FSH + ICSH
Zinc	62 (47–234)	99 (80–230)
Copper	91 (74–111)	86 (59–130)
Barium	66 (41–150)	60 (42–101)

in Table 6, which shows that there was no significant difference between the results by the two assays.

Barium. A portion (2·0 ml.) of 2 M-barium acetate was added to urinary gonadotrophin (about 100 mg.) in 10 ml. of 0·2 M-sodium carbonate in an ice bath. It was added drop by drop with shaking and when the pH had fallen to 7·0 the barium carbonate which had precipitated was removed by centrifuging and the supernatant was treated with about 2 g. of Bio-Deminrolit and filtered. Protein was precipitated by adding 3 vol. of ethanol and then washed with ethanol and ether. About 60% of the original weight of material was recovered in this fraction and the relative potency compared with the original was also about 60% (Table 6). The potency per milligram had not therefore been increased by treatment with barium as in the experiment of Legault-Démare *et al.* (1958).

Reactions with triazine dyes

The dyes Procion Brilliant Red 2BS, Yellow RS and Blue 3G5 were gifts from Imperial Chemical Industries Ltd. (Dyestuffs Division). They all contain the dichlorotriazinyl grouping and sulphonic acid groups. Under the conditions of the following experiments they are believed to be attached to proteins through the amino groups (Hess & Pearce, 1959).

Urinary gonadotrophin (20 mg.) in 2·0 ml. of 0·9% sodium chloride soln. was mixed with 0·2 mg. of triazine dye in 0·5 ml. of 0·9% sodium chloride soln. Protein was precipitated after 30 min. by adding 5 vol. of ethanol. The product was washed with ethanol and ether. Gonadotrophin was recovered after treatment with the blue and yellow dyes with no appreciable loss of biological activity (Table 7) but the recovery with the red dye was poor.

The dyed gonadotrophins were used for electrophoretic experiments on cellulose strips (Oxo Ltd., Medical Department). Horizontal electrophoresis in 0·1 M-sodium acetate containing 0·9% sodium chloride at pH 5·5 was continued for 16 hr. with 0·5 mA/cm. width. Usually a single dyed area was obtained, about 1 cm. on the positive side of the origin, and this was biologically active. Staining with Nigrosine WS (Hopkin and Williams Ltd.) or Lissamine green (Imperial Chemical Industries Ltd.), however, revealed a slower-moving fraction which did not carry the triazine dye and this was also biologically active. These two fractions appeared to be approximately equal in biological activity and no significant differences were found between the results by the two methods of assay. In a typical experiment with the yellow dye the dyed area contained the equivalent of 11·3 (9·0–12·2) mg. of HMG 24 by the uterine-weight assay and 7·8 (3·0–10·8) mg. of HMG 24 by the augmentation method, whereas the second, slower-moving fraction contained 9·5 (8·0–11·3) mg. of HMG 24 and 11·2 (2·0–24·0) mg. of HMG 24 by the same two assays.

On columns of DEAE-cellulose the dyed gonadotrophins were eluted in 0·2 M-ammonium acetate in the same way as untreated gonadotrophins. An appreciable quantity of the dye was retained at the top of the column and this contained no detectable biological activity.

Table 7. *Combination of urinary gonadotrophin with triazine dyes*

Assays for the blue and yellow derivatives were of 2:2 point design and for the red derivatives were 1:2 point design. There was no evidence of lack of parallelism in the dose–response lines and the index of precision (λ) varied between 0·05 and 0·15.

	Potency (% of control with 95% fiducial limits)	
	Augmentation assay for FSH	Uterine-weight assay for FSH + ICSH
Procion blue	106 (63–200)	93 (41–130)
Procion yellow	87 (66–108)	71 (50–100)
Procion red	<50	<50

DISCUSSION

The two biological assays used here have been developed in order to recognize qualitative differences in human urinary gonadotrophins (Brown, 1958, 1959). Borth, Linder & Lunenfeld (1959) suggested that such differences may be due not to changes in the ratio of ICSH to FSH but to other factors such as rates of absorption, toxicity or the presence of synergists or antagonists to gonadotrophin in the extracts. Thus different methods of extraction might give products which appear to contain different ratios of ICSH to FSH but this might be explained by different quantities of impurities contained in each. In the present experiments this possibility does not arise since the treated material was always assayed against gonadotrophin from the same batch. The possibility remains that certain reagents may modify the impurities rather than the gonadotrophin and so alter the relative potency. However, the results obtained with highly purified pituitary fractions are essentially the same as with relatively crude urinary extracts, suggesting that the main effect is on the gonadotrophin rather than on the impurities.

The biological activity of gonadotrophin was altered by reactions which may affect the carbohydrate groups. Oxidation with periodate and methylation with dimethyl sulphate diminished both ICSH and FSH activity. Reduction with

borohydride diminished ICSH more than FSH, as shown by the experiments with crude extracts of urine containing both ICSH and FSH and with purer preparations of human pituitary 'ICSH' and 'FSH'. With urinary extracts the results by the uterine-weight method were consistently lower than those by the augmentation method. With pituitary 'ICSH' the biological activity became undetectable whereas with pituitary 'FSH' the activity was only slightly lowered.

None of the other reactions which were investigated affected the activity of ICSH or FSH differentially. Some of these reactions may be useful, however, as steps in the purification of gonadotrophins. Copper and zinc salts were found to precipitate urinary gonadotrophins with only slight loss of activity. Barium was investigated because it was used successfully for the purification of pregnant-mare serum gonadotrophin by Legault-Démare *et al.* (1958). They obtained a 20-fold increase in potency per milligram but the present experiment showed that no such increase was obtained with human urinary gonadotrophin.

Methylation under the mild conditions used by J. B. Brown (1955) greatly diminished the activity of urinary gonadotrophin. Chemical measurement of the methoxyl groups showed that the treated material contained 1·8%, but it was not possible to show with the available material whether carbohydrate or amino acids had been methylated.

The stability in alkali of the urinary gonadotrophins was investigated to determine whether any changes, particularly qualitative changes, occur during elution in the kaolin-extraction procedure. Gonadotrophin was prepared by the benzoic acid–tungstic acid method since a high pH is not used. This material was quite stable for 20 min. at room temperature at pH 11·5, which is the maximum pH normally used for elution from kaolin. It indicates that no qualitative changes are likely to occur at this stage.

Coloured derivatives of the urinary gonadotrophin with yellow and blue triazine dyes were obtained with satisfactory biological activity. They were prepared so that the behaviour of gonadotrophins in chromatographic and electrophoretic separations could be observed visually. Electrophoresis showed, however, that only a portion of the gonadotrophin carried the dye and there is no biological evidence that the electrophoretic fractions are qualitatively different.

A study of reactions such as those described may eventually lead to a greater knowledge of the chemical nature of those groups in the protein that are necessary for gonadotrophic activity. Quantitative data on the chemical changes would be of small value until pure preparations of gonadotrophins are available.

SUMMARY

1. The effects of various reagents on the activity of human urinary and pituitary gonadotrophins has been studied by two assay methods, the first measuring the combined effects of follicle-stimulating hormone and interstitial-cell-stimulating hormone and the second being more specific for follicle-stimulating hormone.

2. The only reagent to affect one type of activity more than the other was sodium borohydride. This appeared to destroy interstitial-cell-stimulating hormone more readily than follicle-stimulating hormone.

3. Methods are described for the combination of urinary gonadotrophin with zinc, copper and triazine dyes without loss of biological activity.

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Limited Hydrolysis of Levan by a Levanpolyase System

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The metabolism of levans in soil involves a family of levanases (Hestrin & Goldblum, 1953; Fuchs, 1956, 1958, 1959; Loewenberg & Reese, 1957; Ben-Tovim & Hestrin, 1957; Zelikson (Ben-Tovim), 1958; Schlubach, Hohn & Repening, 1959) which deserves further study. Unexpected specificity features distinguish some of these systems.

It is shown in this paper that the activity of one of the systems, 'levanpolyase', is specifically directed towards linkages near branch points in levan. The distribution and types of levanases encountered in a range of micro-organisms are considered. Attention is also directed to the feature that the prior history of cells can exert a regulatory effect on the specificity range of induced fructosidase activities. In this paper oligosaccharides in which a reducing fructose moiety bears hexosyl or polyhexosyl at carbon atom 1 or 6 will be designated as 1-R- or 6-R-fructose respectively.

METHODS AND MATERIALS

The levan used was prepared from a sucrose culture of *Aerobacter levanicum* as described by Feingold, Avigad & Hestrin (1956), except as otherwise is specified.

Isolation and characterization of strain 7

The medium contained (%): levan, 1·0; $MgSO_4,7H_2O$, 0·05; K_2HPO_4, 0·1; peptone, 1·0; yeast extract, 0·3; pH 7·2. It had been sterilized by heating three times successively for 1 hr. in an open autoclave. After seeding with a sample of Jerusalem soil, the mixture was incubated for 3 days at 30° and plated on to similar medium with 2·5% of agar. Inocula from isolated colonies were transferred to fresh medium and ethanol was then added to the original plate. The appearance under the ethanol of a transparent halo around a colony demonstrated that an extracellular system catalysing cleavage of levan had been secreted.

Strain 7 proved to be a strict aerobe. The cells were Gram-positive in young cultures only. They were pleomorphic, with beads, metachromatic, swollen, 0·5–0·8 μ in diameter and were poorly motile. Growth was optimum at 30°. Colonies on nutrient agar were whitish in colour and opaque; they were round, smooth and convex in form. There was no growth with ammonium sulphate as sole nitrogen source or with citrate as sole carbon source; there was no production of H_2S; indole production failed; the methyl-red test was negative; the catalase test was positive; there was reduction of nitrates to nitrites; gelatin was slowly liquefied; litmus–milk was decolorized (with slow peptonization) after 3 days; growth on potato extract (with or without glucose) was abundant, creamy-white and glistening. Starch was hydrolysed. Cellulose was not attacked even after 20 days. Several carbohydrates (D-series: glucose, galactose, fructose, mannitol, sorbitol, dulcitol, arabinose, xylose; L-series: arabinose, rhamnose; saccharides: sucrose, maltose, lactose, cellobiose, methyl α-glucoside, salicin and inulin) gave slow acid production without gas evolution. The following gave neither acid nor gas: D-mannose, glycerol, starch and dextrin. These properties place the organism within *Arthrobacter* in Bergey's classification (Breed, Murray & Smith, 1957), and they depart only in minor features (catalase test; pigmentation on potato extract; litmus–milk test) from those of *Arthrobacter tumescens*.

The organism was maintained on slants of nutrient agar (Difco Laboratories, Detroit, Mich., U.S.A.). The interval between successive transfers was 90 days (2 days at 30° followed by 12 weeks at 6°).

Preparation of enzyme

Extracellular system. A culture (1-day-old slant) of strain 7 was washed into a Roux flask containing solidified levan medium under a layer of liquid medium at 30°. Activity of extracellular levanpolyase in the liquid layer reached a peak after 2 days. Liquor from 3-day cultures was cleared by centrifuging and brought to saturation with ammonium sulphate (pH 7·0–7·2; 6°). The precipitate was dissolved in five-times-diluted McIlvaine phosphate–citrate buffer, pH 7·0 (Gomori, 1955), and the solution was brought to 60% saturation with ammonium sulphate; a precipitate which formed at this point was discarded. At saturation with ammonium sulphate, a further precipitate formed and was packed down by centrifuging. Under standard conditions of assay (see below), it hydrolysed 11 μmoles of fructocarbonyl linkages in levan/mg. of protein/hr. The recovery of the original activity was essentially complete (> 80%), activity related to protein content (Lowry, Rosebrough, Farr & Randall, 1951) being about five times that of the raw liquor. The material was stored at −12° without loss of activity even after 12 months of storage. On the day of an experiment, the frozen preparation was thawed and dissolved in water. Final enzyme concentration in a standard reaction mixture was made to be about three times that of the enzyme in the original culture medium. Residual ammonium sulphate in the standard system was < 2% and without evident influence on the course of the reaction.

Homogenate. Cells (140 mg. of protein) were washed, mixed with Ballotini beads (7 g.) in phosphate–citrate solution, pH 7·0 (7 ml.), and the mixture was exposed to two treatments in a Nossal shaker for 30 sec. Debris was removed finally by centrifuging (12 000 *g* for 15 min.).

Strains other than 7

Procedures as developed for strain 7 were also used in experiments on other levanase-forming strains.

We failed in repeated attempts to isolate levanase-forming *Azotobacter* strains directly from soil by enrichment in levan medium (Beijerinck, 1912). Such were readily found, however, among soil isolates that had been grown in mineral solution (K_2HPO_4, 0·1%; $MgSO_4$, 0·05%; $FeCl_3$, 0·03%; Na_2MoO_4, 0·001%; $CaCl_2$, 0·01%; tap water; pH 7·2–7·4) on 1% ethanol or on 2% glucose and 2% $CaCO_3$.

Examination of enzyme activity

A solution of levan was prepared at room temperature by stirring dry powdered levan into a small volume of water and adding more water gradually.

A standard reaction mixture contained levan (2%) in McIlvaine phosphate–citrate buffer, pH 6·0 (diluted 1:20 in the final system), and enzyme solution (0·1 mg. of protein/ml. of final reaction mixture) under toluene. The temperature was 30°. Toluene did not affect the enzyme activity.

Measurement of products of hydrolysis. Total anhydrofructose was determined colorimetrically as by Roe, Epstein & Goldstein (1949).

Reducing power was measured in a copper–phosphate reagent (Somogyi, 1945; Nelson, 1944) with a heating time of 15 min. In these conditions, the molar reducing powers of fructose, of members of polymer series of the type 1-R-fructose and of members of polymer series of the type 6-R-fructose towards this reagent were 1·00:1·00:0·89. The molar reducing powers were independent of the size of R within wide limits [degrees of polymerization (n) 2, 3, ..., 8]. It was possible to demonstrate the above relationship in all of the following: (*a*) fructosides recovered from partial acid hydrolysate of inulin; (*b*) fructosides recovered from partial acid hydrolysate of levan; (*c*) fructosides (1-R- and 6-R-fructose series respectively) recovered from products of action of levanpolyase on levan.

Components of type 6-R-fructose were assayed specifically in the presence of 1-R-fructose by reaction with 2:3:5-triphenyltetrazolium reagent (Avigad, Zelikson & Hestrin, 1961). The reagent was calibrated against a known levanbiose solution.

For mixtures containing no free fructose in which 6-R-fructose and 1-R-fructose were equimolar, the percentage hydrolysis of levan was calculated from the copper-reducing power by multiplying the fructose equivalent by a factor (1·05) which corrected for the circumstance that 6-R-fructose exhibits a lower reactivity towards the copper reagent than does an equimolar amount of 1-R-fructose or free fructose. 1-R-Fructose was obtained by deducting 6-R-fructose from total free fructocarbonyl.

Measurement of rate of enzymic hydrolysis. Enzymic hydrolysis of levan could readily be followed for up to 1·00% of total hydrolysis by observing the decline of the extinction of the solution (Goldblum, 1953; Fuchs, 1959). A Klett photoelectric colorimeter set at filter 540 was used. The turbidity of enzyme (blank) was constant throughout the reaction time and equivalent to about 7% of that of levan in the standard system. Levan solution in the absence of enzyme showed no change in turbidity during 24 hr. Enzymic hydrolysis of levan to an extent of 1·00% (on the basis of reducing power) corresponded to a 60% decline in turbidity. Within this limit the turbidity decreased in a linear manner with time and was directly proportional to degree of hydrolysis (estimated by reducing power) irrespective of whether the catalyst system employed was an acid or levanpolyase.

Fructosides

Enzymic hydrolysates of levan were resolved crudely by fractionation with ethanol. The reagent was added to the original mixtures at room temperature with continuous stirring, the precipitate being collected after 5 min. by centrifuging at 8000 *g* for 15 min. Salts present in the hydrolysates markedly influenced the solubility of levulans in this water–ethanol system. The fractionation gave a pattern in conformity with the following (ethanol–water, v/v): $n > 1000$ (2 vol.); n 20–1000 (2–4 vol.); n 10–20 (4–6 vol.); n 8–10 (6–8 vol.).

Oligofructosides ($n < 12$) in partial hydrolysates of levan and inulin were resolved by paper chromatography (for details of solvents, etc., see Table 1 in Hestrin & Avigad, 1958) and were recovered from the paper by elution with water and freeze-drying.

Inulin was a commercial specimen from dahlia tubers (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.).

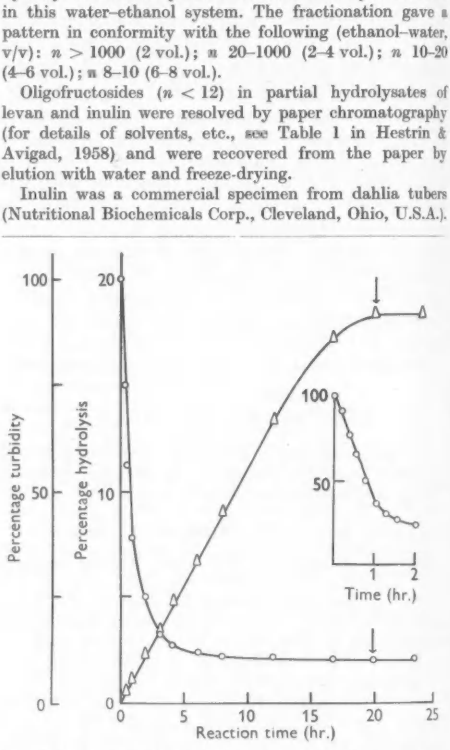

Fig. 1. Kinetics of hydrolysis of levan by levanpolyase. Standard reaction mixture was used. △, Percentage hydrolysis; ○, percentage turbidity. Inset shows the linear course of the initial decline of turbidity. The arrows indicate an addition of fresh enzyme. Such addition failed to cause a reaction burst, presumably because all susceptible linkages had by this time already been cleaved. The initial turbidity value plotted as 100 corresponded to 260 Klett units and was in a range in which turbidity and levan concentration are directly related. Addition of fresh levan, rather than of fresh enzyme at the time marked by the arrows, resulted in a renewed reaction, whose rate was about 60% of the original rate and which proceeded to a limit similar in magnitude to that of the original reaction.

RESULTS

Experiments on extracellular levanpolyase formed by strain 7

Course of reaction. The action of levanpolyase on levan was manifested by a decrease in turbidity and by release of reducing groups. It can be seen in Fig. 1 that the reaction proceeded to a limit at 18·6% of total hydrolysis. The decline of turbidity was complete long before the release of reducing groups had ceased. The course remained zero order in time throughout at least 90% of the total reaction. Variation in either the enzyme concentration (Fig. 2) or in the concentration of levan within a wide tested range [0·5–4·0% (w/v) of levan] did not alter the reaction limit. The limit was not one determined by enzyme inactivation, since renewal of the enzyme supply failed to abolish the limit. Moreover, it was not simply an effect of inhibition by product, since addition of a fresh supply of levan brought about a renewed reaction which again proceeded to the same limit.

Composition of product formed from levan. The limit-hydrolysate consisted of fructosides ranging in n continuously from 2 to at least 12. No free fructose was found. About 75% of the material was in the n range 7–12. Components with n 13–20 were a small amount; no component with $n > 20$ was detectable.

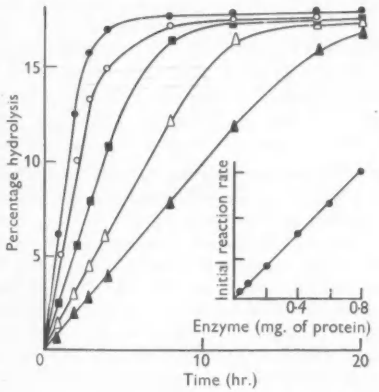

Fig. 2. Influence of enzyme concentration on rate and limit of levan cleavage. Standard reaction system was used with varying enzyme concentration (mg. of protein/ml. of final reaction mixture): ●, 0·80; ○, 0·60; ■, 0·40; △, 0·20; ▲, 0·10. In the inset, relative enzyme concentration is plotted against the initial reaction rate. Throughout the examined range, the initial rate of reaction was directly proportional to the enzyme concentration and the reaction limit remained a constant.

The products constituted two apparently continuous polymer homologous series: A and B (Figs. 3 and 4; Table 1). The members of the A series reduced copper but not tetrazolium and ran more rapidly on the paper than the corresponding members of B series. Members of B series reduced tetrazolium as well as copper. On the basis of the reducing properties of the compounds, it must be concluded that the A series conform to type 1-R-fructose, whereas the B series can be assumed to be of type 6-R-fructose. The chromatographic mobilities of the B series correspond to that of the polymer

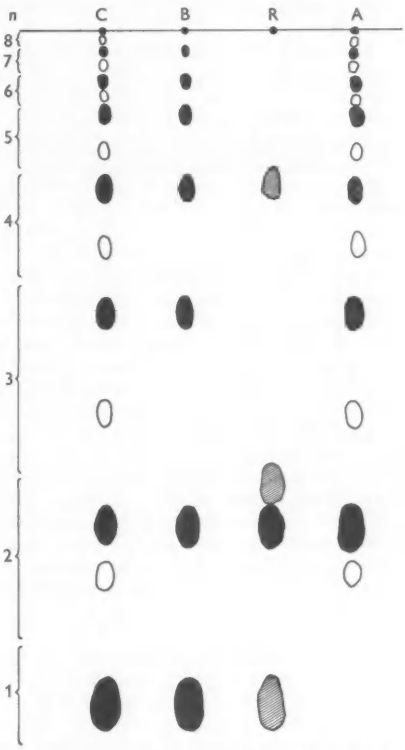

Fig. 3. Chromatographic analysis of partial hydrolysates of levan. Circled and blocked regions designate respectively compounds of the type 1-R- and 6-R-fructose. Hatched regions designate sugars of other types. n, Degree of polymerization. Solvent system and spray reagents are as given in Table 1. A, Levan hydrolysed to limit by levanpolyase (standard reaction mixture); B, levan partially hydrolysed by acid (hydrolysis at pH 2·2 in dilute sulphuric acid for 1 hr. at 60°); C, levan hydrolysed to limit by modified levanpolyase (fructogenic enzyme type); R, known reference sugars: fructose, levanbiose, sucrose, raffinose, levan (in order of decreasing mobility).

Table 1. *Products of hydrolysis of levan by levanpolyase*

Standard reaction mixture, in which hydrolysis of levan had proceeded to limit, was analysed at 20 hr. Values of R_{Fru} are for the solvent system propan-1-ol–ethyl acetate–water (7:1:2, by vol.) (Albon & Gross, 1952). Paper chromatograms were treated with urea–phosphoric acid (Wise, Dimler, Davis & Rist, 1955) to locate ketose, and with alkaline triphenyltetrazolium chloride (Wallenfels, 1950; Feingold, Avigad & Hestrin, 1956) to locate 6-R-fructose. Quantities of carbohydrate present at different positions in a chromatogram were assayed in aqueous eluates prepared from appropriate sections of the paper.

Component	R_{Fru}		Mass (% of total levan)		
	1-R-Fructose series (A)	6-R-Fructose series (B)	1-R-Fructose series (A)	6-R-Fructose series (B)	A + B*
1	1·00	1·00	.	.	0·02 (20·0)
2	0·82	0·72	0·2	1·0	1·2
3	0·61	0·47	0·5	2·5	3·0
4	0·39	0·24	1·0	4·0	5·0
5	0·20	0·13	2·0	4·0	6·0
6	0·10	0·07	3·5	4·0	7·5
7	0·050	0·035	4·0	4·0	8·0
8	0·022	0·016	.	.	.
8†	.	.	33·0	35·0	68·0 (47·0)

* Values in parentheses are data obtained with a 'fructogenic' levanpolyase system (see Results section).
† Fraction precipitated from original reaction mixture on addition of 8 vol. of ethanol.

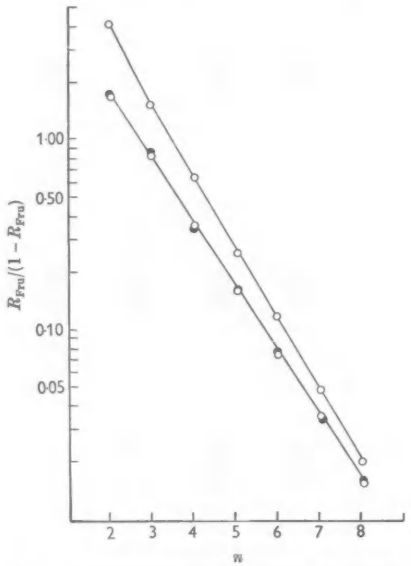

Fig. 4. R_{Fru} of constituents of limit hydrolysate of levan. Values are from Table 1. Degree of polymerization (n) was obtained from the ratio 'total fructose/reducing power'; '$R_{Fru}/(1-R_{Fru})$' values are plotted on a logarithmic scale (French & Wild, 1953). Slopes are 1·67 for the A and 1·52 for the B series of homologous polymers. ○, Fructosides obtained from limit hydrolysis of levan by levanpolyase; ●, fructosides obtained from partial acid hydrolysate of levan.

Table 2. *Relative proportion of 1-R- and 6-R-fructose in levan hydrolysate*

Standard reaction mixture was used.

Reaction time (hr.)	Hydrolysis (%)	Composition of hydrolysate (molar, relative to total anhydrofructose as 100)		
		6-R-Fructose (a)	1-R-Fructose (b)	$(a)/(b)$
0	0·0	0	0	—
6	6·6	3·4	3·2	1·07
20	18·6	9·5	9·1	1·05

series obtained from levan by acid hydrolysis, a series which must be presumed, for low values of n, to consist of linear levulans.

Digests at 50 % of limit reaction gave chromatograms which resembled qualitatively those from digests at limit-reaction (Fig. 3). The mixtures contained no free fructose. At an initial stage (6·6 % of total hydrolysis), as at the limit of reaction (18·6 % of total hydrolysis), 1-R- and 6-R-fructose contributed on an equimolar basis to the total free fructocarbonyl (Table 2).

Weights of product in the limit hydrolysate increased with degree of polymerization, approaching a limit at n 6 both in the A and B series (Table 1).

Ratio '6-R-fructose/1-R-fructose' in the limit hydrolysate decreased with degree of polymerization up to about n 7, ranging from > 5 (at n 2) to ~ 1 (at n 7 and for $n > 7$) (Table 1).

Influence of pH. Optimum pH activity was found at 5·8 (Fig. 5). Throughout the range in which any activity was demonstrable (pH 4·5–7·4) the products of reaction (as resolved chromatographically in mixtures at both partial and limit hydrolysis) were identical, and the ratio 'initial rate of decline of turbidity/initial rate of release of reducing groups' remained constant.

Although this enzyme system exhibited negligible loss of activity during 20 hr. at 25° at pH 6·0–7·4, it lost almost all activity at < pH 5·0 within 5 hr.

Levan concentration. In the conditions of the standard reaction, the reaction velocity varied directly as the initial levan concentration over a wide range (0·5–4·0 %).

Substrate specificity. The following substrates (which are readily cleaved by yeast invertase) were not hydrolysed or disproportionated by the enzyme: sucrose; raffinose; inulobiose and its polymer homologues, n 3–7; levanbiose and its polymer homologues, n 3–7; products (A and B series, n 2–8) formed from levan by levanpolyase.

Substrates that were hydrolysed were (% hydrolysis limit values are given in parentheses): levans formed in bacterial cultures from sucrose, *Aerobacter levanicum* (18·6 %), *Bacillus polymyxa* (19·5 %), *Arthrobacter* 7 (1-day culture) (20·7 %). The levan formed from sucrose by an homogenate of *Arthrobacter* cells gave a similar hydrolysis limit (21·5 %). The products resembled that from *Aerobacter levanicum* levan (Fig. 3A), but contained in addition a small amount of free fructose (1 % of total initial levan).

Inulin was converted into products which all had $n > 5$. They comprised n 6, 7, 8 and 9 (in amounts increasing with n) and a relatively large amount of $n > 9$. The initial rate of hydrolysis in inulin was about one-tenth of that in levan; the hydrolysis limit was only 3·0 %. Had cleavage occurred in the sucrose terminal of inulin, glucose should have been liberated, but such was not the case (glucose liberation at limit of reaction: < 0·1 % of initial inulin).

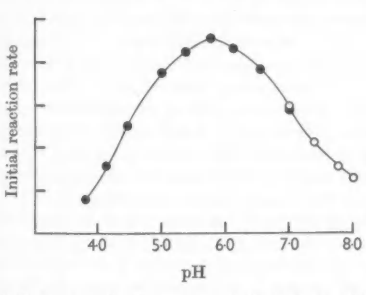

Fig. 5. Influence of pH on enzymic hydrolysis of levan. Standard reaction mixture was used. pH was adjusted with sodium phosphate–sodium citrate (McIlvaine) buffer (diluted 1:20 in final mixture) in the range pH 3·8–7·0 (●), and with sodium phosphate (0·01 M-phosphate) in the range pH 7·0–8·0 (○). Reaction time was 15 min. Enzyme activity decreased < 5 % within this time. The initial reaction rate was determined turbidimetrically.

Effect of carbohydrate in growth medium. The formation of extracellular levanpolyase in strain 7 was completely suppressed by growth on glucose or fructose. However, an intracellular system which hydrolyses oligofructosides (sucrose, raffinose) proved to be constitutive in this organism. Ranges of fructosidase activities evoked in strain 7 by appropriate inducers are compared in Table 3.

A hydrolysis of sucrose or raffinose by an enzyme preparation containing levanase might occur via intermediary production of levan if the system in question also contains levansucrase. Sucrose- and raffinose-hydrolysing systems described in Table 3, whose action on levan resulted in a total conversion of the latter into fructoses, might indeed be examples of hydrolysis involving such an enzyme mixture.

The inulin-induced systems (intra- and extracellular) hydrolysed both levan and inulin totally. Whether levanpolyase itself had been induced in the bacteria by inulin and participated in the observed total hydrolysis of levan is unknown.

Influence of history of cells. Cells (strain 7) were carried for three successive transfers on nutrient agar, the interval between transfers being 7 days (1 day at 30° and 6 days at room temperature) rather than 90 as in our standard procedure. The extracellular system induced by levan in such cells was fructogenic and gave as end products of reaction on levan both a large amount of fructose and in addition the series A and B. The limit-hydrolysis value of levan was 39·7 % as against 18·6 % in the standard system. The elevation in limit value could be accounted for in terms of selective fructose release from components in the range $n > 7$ (see Table 1). The 'fructogenic' levanpolyase system failed to hydrolyse fructosides (A and B series) $n < 7$, but caused a 30 % hydrolysis into fructose of a fraction $n > 7$ that had been formed from levan by standard levanpolyase. Furthermore, this fructogenic system totally converted inulin into fructose and also hydrolysed sucrose and raffinose.

An analogous dependence of a response pattern on cell history was encountered in the course of tests with a species of *Pseudomonas* (strain 3). This strain excreted a typical non-fructogenic levanpolyase system in inulin medium after maintenance on nutrient agar for 90 days. It gave a fructogenic levanpolyase system on inulin medium after three 7-day transfers on nutrient agar.

Table 3. *Influence of carbohydrate in medium on substrate range of fructosidase systems induced in strain 7*

Carbohydrate in medium: 2%. Activities tabulated are as found after 3 days of growth. Activity in extracellular fraction was concentrated in every case by fractionation with ammonium sulphate (see Methods section). Approach to the limit of hydrolysis was confirmed in every case by adding fresh enzyme to a reaction mixture in which hydrolysis had reached a constant value. Reaction of total hydrolysis yielding fructose as sole end product was in every case characterized by an intermediary appearance of a series of oligofructosides (*n* 2, 3, ..., 8). +, Hydrolytic cleavage of interfructosidic linkage in substrate; −, absence of detectable hydrolytic activity.

Carbohydrate in growth medium	Enzyme system	Hydrolysis limit (%) In levan	Hydrolysis limit (%) In inulin	Hydrolysis of fructosidic linkage in sucrose and raffinose	End products formed From levan	End products formed From inulin
Hexose (glucose, fructose)	Extracellular	—	—	−	.	.
	Cell homogenate	—	—	+	.	.
Sucrose*	Extracellular	39·5†	100	+	Series A and B and fructose	Fructose
	Cell homogenate	100†	100	+	Fructose	Fructose
Inulin	Extracellular	100	100	+	Fructose	Fructose
	Cell homogenate	100	100	+	Fructose	Fructose
Levan	Extracellular	18·6	3·0	−	Series A and B	*n* 6, 7, 8 and ⩾ 9
	Cell homogenate	>50‡	100	+	Series A and B and fructose	Fructose

* With cells on sucrose medium there was an appearance of macromolecular levan in the medium on the first day. This product was eventually degraded, being replaced at the third day by oligofructosides (series A and B) and free fructose.

† Extracellular enzyme from first day of growth on sucrose exhibited a hydrolysis limit with levan of only 4·0%, the end products of reaction being levanbiose and a macromolecular fructan. The corresponding cell-homogenate system at this time gave with levan a similar hydrolysis limit and the same end products but with a trace of fructose.

‡ Limit of hydrolysis was not reached in this experiment.

To ascertain whether the altered pattern of response to inducer is reversible, strains 7 and 3, after they had been modified as described in respect of response to inducer, were maintained on nutrient agar for 90 days without intervening transfer. Both strains then reverted to their original response pattern, namely to production of extracellular non-fructogenic levanpolyase in response to inducer.

Still another bacterial strain (*Micrococcus* 16), on the other hand, consistently gave a fructogenic extracellular levanpolyase system when grown on levan, even though prior transfers of this strain in nutrient agar had been made at the 90-day interval, which in strains 7 and 3 had led to suppression of fructogenic response.

Distribution of levanpolyase and other levanases among micro-organisms

Earlier investigators [Beijerinck, 1910*a*, *b*, 1912; Hestrin & Goldblum, 1953; Fuchs, 1956; Ben-Tovim & Hestrin, 1957; Loewenberg & Reese, 1957; Zelikson (Ben-Tovim), 1958; see in particular the comprehensive recent monograph of Fuchs, 1959] have noted the wide distribution of ability to utilize levan in micro-organisms. Among isolates from soil studied in this laboratory, the ability to degrade levan was encountered in the following (numbers in parentheses designate the number of species and types): *Bacillus* (5), *Azotobacter* (2), *Arthrobacter* (2), *Micrococcus* (2), *Pseudomonas* (3), Gram-negative rods (4) and yeast (3).

The extracellular occurrence of levanase was a common feature in these species provided that a suitable inducer was present in the growth medium. Glucose consistently failed to induce extracellular levanase. In all but one instance, cells responding to levan with formation of extracellular levanase also responded in this manner to sucrose and inulin. In *Azotobacter* strain 58, however, the response occurred with sucrose and levan but not with inulin.

There was a marked similarity of properties in the levanpolyase system excreted by a wide range of bacteria, including the following (the numeral indicates the serial number of the strain; growth sugar is given in parentheses): *Arthrobacter* 7 (levan); *Pseudomonas* 3 (inulin); *Pseudomonas* 32 (levan); *Bacillus* 30 (inulin, levan); *Azotobacter* 58 (levan). The levanpolyase system in all these cases exhibited a hydrolysis limit of 19% in levan and a limit of 3·0% in inulin. Enzyme formed no fructose but only oligofructosides ranging in *n* from 2 to at least 12 as end products of levan hydrolysis and oligofructosides of $n \geqslant 6$ as end products of inulin hydrolysis. The time required for 1% levan hydrolysis was in every case the same as that for 60% decrease of turbidity. Sucrose and raffinose were not hydrolysed by any of these systems. The pH–activity function of levan hydrolysis was the same throughout as in *Arthrobacter* (Fig. 5).

In another group of experiments, a levanase system excreted by cells was characterized by ability to effect total conversion of levan and inulin into fructose and by an ability also to hydrolyse sucrose and raffinose as well as members of oligolevan series A and B. Such a system was obtained from the following (the number indicates the serial number of the strain; growth sugar is given in parentheses): *Bacillus polymyxa* (sucrose, inulin, levan); *Arthrobacter* 7 (inulin); *Azotobacter* 53 (sucrose, inulin, levan). The optimum pH of levan hydrolysis in every such case was at 5·8–6·0. Whether a total hydrolysis of levan into fructose should be ascribed to an action of levanpolyase operating in conjunction with other carbohydrases remains to be clarified.

A different type of limited levan hydrolysis has been afforded by an extracellular-enzyme system formed by a species of *Pseudomonas* (strain 11) during growth on levan, inulin or sucrose. In this system, the hydrolysis limit was in the range 5·0–8·0 %, and the products of reaction were exclusively fructose or levanbiose or both, together with a high-polymer product. Details of this system, which recalls that found extracellularly in 1-day-old culture of *Arthrobacter* 7 on sucrose (Table 3), will be given separately.

Still another *Pseudomonas* (strain 8) gave an extracellular system which catalysed a limited cleavage (3·0 %) in levan without attendant release of any product with $n < 8$.

DISCUSSION

It has been suggested that bacterial levan has a branched structure in which the ratio of the principal interfructosidic linkage (type $2 \rightarrow 6$) to branch linkage (type $2 \rightarrow 1$) is typically about 9 (Bell & Dedonder, 1954; Hestrin, Feingold & Avigad, 1956; Feingold & Gehatia, 1957). Further evidence for this view is afforded by the present findings that hydrolysis of *Aerobacter levanicum* levan by levanpolyase yields products of the type of 1-R-fructose and that the molar yield of the latter corresponds at the limit of this enzymic hydrolysis to the branching degree as obtained by methylation analysis.

The question arises whether levanpolyase could be used in a routine manner and on a microscale to estimate branching degree in levans. Such use of a levanpolyase preparation would have to be predicated upon the following assumptions, which have yet to be proved rigidly: (1) that this enzyme acting on levan neither breaks nor makes a $2 \rightarrow 1$ linkage; (2) that no $2 \rightarrow 6$ linkage involving levanfructose units bearing a substituent on C-1 escapes cleavage by the enzyme.

Since the molar yields of 1-R- and 6-R-fructose released from levan by levanpolyase are equal and in one-to-one correspondence with the estimated amount of $2 \rightarrow 1$ linkage, levanpolyase can be considered as having split two $2 \rightarrow 6$ linkages in levan for every $2 \rightarrow 1$ linkage present. One-half of all the susceptible $2 \rightarrow 6$ linkages have been shown to be at C-2 of fructose units which bear a substituent at C-1 (Fig. 6). As to the remaining susceptible $2 \rightarrow 6$ linkages, it is both uncertain whether they are in primary or secondary chains (i.e. in chains respectively on C-6 or C-1 of a disubstituted fructose unit) and whether their distance from the nearest $2 \rightarrow 1$ linkage is a constant or a variable.

Salient features of the make-up of the mixture of end products formed from levan by levanpolyase are the following: (*a*) the products fall into polymer series in which all possible values of n in the range 2 to at least 12 are represented; (*b*) at each successive level of n in the mixture of products, both 1-R- and 6-R-fructose occur; (*c*) the overall ratio '1-R-fructose/6-R-fructose' is near 1·0; (*d*) the limit percentage of hydrolysis is a value (18·6 %) which is twice the degree of branching estimated by methylation analysis; (*e*) the ratio '$2 \rightarrow 1$ linkage/$2 \rightarrow 6$ linkage' in the mixture of products is as in levan. To accommodate these findings, it can be suggested that the levanpolyase protein tends to be selectively bound to levan at structural anomalies (branch points or chain terminals or both) and that the hydrolytic function of protein bound in

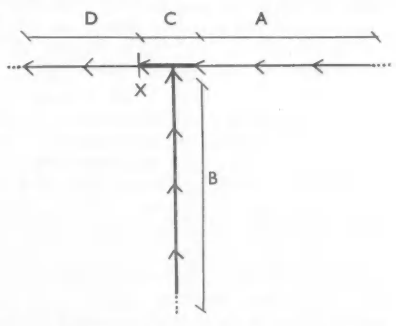

Fig. 6. Scheme of linkage positions in levan that are susceptible to cleavage by levanpolyase. The scheme shows a branched part of a levan molecule. $2 \rightarrow 6$-linked anhydrofructose units are represented by arrows in end-to-end arrangement. A secondary chain (B) is attached to C-1 of an anhydrofructose unit (C). A primary chain (A) is on C-6 of this unit, which is linked glycosidically via its C-2 to the remainder (D) of the levan molecule. Levanpolyase splits two $2 \rightarrow 6$ linkages per $2 \rightarrow 1$ linkage in the molecule. One $2 \rightarrow 6$ linkage out of each two linkages split by the enzyme involves C-2 of the disubstituted fructose unit (C) and is designated by X.

this manner can bear only on particular linkages in the levan chain.

The range of n in the end products formed from levanpolyase could be a reflexion of a variability of either an internode distance (terminal-to-branch distance or branch-to-branch distance or both) in levan or of a distance between a binding site and the nearest cleavage site. Taking either of these distances as a constant, the distribution range and pattern of the other can be expected to be mirrored in the values of n found in the end products. It has been shown that these products range continuously in n from 2 fructose units to at least 12, and do not exceed 20, and that there is a distinct maximum in the range of 9 ± 2.

The mode of the action of our levanpolyase preparations on inulin needs elucidation. The degree of polymerization for inulin being about 35 fructose units (Hirst, McGilvary & Percival, 1950), a hydrolysis limit of 3·0% as observed in inulin would correspond to one cleavage per chain. Since this reaction did not release any glucose, the susceptible linkage could not be in the sucrose configuration at the glucose terminal. Since the end products of hydrolysis all had $n > 5$, it can be suggested further that the susceptible linkage was at least 6 fructose units removed from any chain end. The fact that the reaction products all had $n > 5$ implies incidentally that a large fraction of the overall weight of the substrate preparation must have been involved in the observed transformation, and thus a cleavage of any minor contaminant of the inulin preparation could not by itself account for the observed effect. It remains undetermined whether the same enzyme entity which effected the cleavage of particular $2 \rightarrow 6$ linkages in levan was that which hydrolysed a $2 \rightarrow 1$ linkage in inulin. To account on a tentative basis for the finding that all end products of the inulin hydrolysis had $n > 5$ and that they comprised compounds with n equalling 6, 7, 8, 9 and > 9, we might assume that this enzyme can be effectively bound by the original inulin chains but not by any product of their cleavage, that such binding occurs at a fixed distance in respect to the chain ends and that when the protein is bound in this manner its hydrolytic function can be made to bear only on linkage positions that lie within a particular distance range (e.g. 6 to about 9 fructose units) from the chain end.

It has been noted that in the levan–levanpolyase system the release of reducing groups continues well beyond the time point at which the decline in turbidity is complete. Commonly, in substrate–enzyme systems, substrate molecules outnumber the enzyme molecules by several orders of magnitude. In such systems, a kinetic feature such as that noted above would be an indication in favour of a multi-chain type of attack. With the levan–levanpolyase system, however, the molecular weight of the substrate greatly exceeds that of most known enzymes and is of the order of 10^7 (Hestrin *et al.* 1956; Feingold & Gehatia, 1957). For this case therefore it is not valid to assume without proof that the system contains a large excess of substrate molecules over enzyme molecules. For this reason, the question whether levanpolyase acts via a multi-chain or single-chain mechanism cannot be decided on the basis of the available kinetic information.

Where an enzyme preparation of levanpolyase affords free fructose in addition to 1-R- and 6-R-fructose as end products of levan hydrolysis, it seems reasonable to make the assumption that levanpolyase is in this case accompanied by an enzyme entity which is without action on oligolevans but effectively hydrolyses a $2 \rightarrow 6$ linkage one fructose unit away from the ends of levan chains which exceed a certain minimum size. A fructogenic component of this type has been found to be selectively sensitive to inhibition by heavy metals (Ben-Tovim & Hestrin, 1957). The appearance of the fructogenic component in the extracellular phase of a bacterial culture is probably associated with a condition of leakiness in the bacterial cell wall, since such a component has been detected in the homogenate of cells, even when it could not be found in the extracellular phase of the culture (see Table 3).

The ability of a levanpolyase preparation to give rise to fructose together with oligolevans among the end products of levan hydrolysis has been found to be completely linked with ability to hydrolyse sucrose and raffinose and to hydrolyse inulin totally to fructose. Since this system nevertheless did not hydrolyse oligolevans, it cannot be regarded as a universal β-fructofuranosidase of the type of yeast invertase.

To account for the several patterns of levan hydrolysis observed in the course of the present investigation, the occurrence of three or more types of levanase may be postulated: (1) the system 'levanpolyase', which converts levan into a twin polymer series (1-R- and 6-R-fructose) of oligolevans; (2) the levanase (obtained from *Pseudomonas* strain 8 grown on levan), which only splits linkages in levan which are at a relatively large distance from the chain ends and hence affords only high-molecular-weight products ($n > 8$); (3) the levanase (obtained from *Arthrobacter* strain 7 grown on sucrose and from *Pseudomonas* strain 11 grown on levan or sucrose), which appears to split only linkages in outer chains and converts levan into levanbiose and a high-polymer product. Joint action of a specific levanase together with an oligolevanase could account for observed total cleavages

of levan into fructose. Whether the levanase family also includes proteins of wide substrate range which can act both on native levan and on any product of its partial hydrolysis remains to be determined.

SUMMARY

1. Enzyme systems which effect the hydrolysis of a bacterial levan were adaptively formed by a range of soil micro-organisms.

2. There were at least three distinct enzyme types in this group:

(*a*) A system ('levanpolyase') which converted levan into end products consisting of two homologous polymer series (1-R- and 6-R-fructose) ranging continuously in n from 2 to at least 12, with a maximum in the range 9 ± 2.

(*b*) A system which converted levan into end products all of which had $n > 8$.

(*c*) A system whose action on levan gave only levanbiose and a high-molecular-weight product.

3. Exhaustive action of the levanpolyase system on levan gave 1-R- and 6-R-fructose in equimolar amounts that were the equivalent of the branching degree of levan as estimated by methylation analysis. It can thus be inferred that susceptible linkages in levan were all of the type $2 \rightarrow 6$, and that one-half of these were at C-2 of fructose units which have a substituent on C-1. Sucrose and raffinose were not cleaved by this system, whereas inulin was hydrolysed to an extent of only 3·0% and gave end products which all had $n > 5$.

4. Whereas typical preparations of levanpolyase formed no free fructose from levan, inulin or sucrose, cells grown on levan after frequent prior transfers on nutrient agar gave an enzyme system which did hydrolyse sucrose, hydrolysed inulin totally and afforded both fructose and a twin-polymer series (1-R- and 6-R-fructose) among the end products formed from levan.

5. A hypothesis is proposed which may account for the observed differential activity of levanpolyase on apparently similar linkages within the structure of a levan.

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Preferential Inhibition of Phosphorylation in Different Parts of the Respiratory Chain in Mitochondria Obtained from Animals Injected with Cobra Venom

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Previous studies have demonstrated that the phospholipase A from cobra venom can uncouple oxidative phosphorylation in brain and liver mitochondria (Aravindakshan & Braganca, 1959). These effects had been observed both in experiments *in vitro* as well as in animals injected with the material. Comparative studies carried out with animals injected with crude cobra venom and with heated venom (phospholipase A) had shown that heated venom could not lower the P:O ratio in the intact animal to less than 1·5, even if the animals were injected with large quantities of the material or killed after longer intervals of time. It is well established that three molecules of adenosine triphosphate are formed during the transfer of electrons from diphosphopyridine nucleotide-linked substrates to oxygen. The three phosphorylations are known to occur in steps in different regions of the respiratory chain (Chance & Williams, 1956). It was therefore of interest to explore whether the partial uncoupling of oxidative phosphorylation observed in animals injected with preparations containing phospholipase A was confined to a particular site. Experiments *in vitro* designed to localize the site of activity of some other uncoupling agents have shown that gramicidin (Lehninger, 1954–55) and salicylate (Penniall, 1958) preferentially inhibit the phosphorylation in the region of cytochrome *c*–oxygen. The effect of thyroxine has been investigated in the intact animal by Maley & Lardy (1955), and the data show that thyroxine is equally effective in uncoupling the various phosphorylation steps.

The evidence described in this paper demonstrates that phospholipase A (heated venom) can preferentially uncouple the phosphorylation coupled to cytochrome oxidase when added to isolated brain or liver mitochondria as well as in animals injected with the substance. Apparently in the intact animal the phospholipids associated with phosphorylation in the region of reduced diphosphopyridine nucleotide–cytochrome *c* are not easily attacked by phospholipase A.

EXPERIMENTAL

Inorganic phosphorus was determined according to Lowry & Lopez (1946). Hexokinase was prepared from baker's yeast by the method of Berger, Slein, Colowick & Cori (1946). Heated cobra venom (*Naja naja*) was prepared by heating an aqueous solution (pH 6) of crude venom at 100° for 15 min. This was employed as the source of phospholipase A as recommended by Braganca & Quastel (1953). The quantity of heated venom employed is expressed in terms of the weight of crude venom from which it was obtained.

The P:O ratio in the reduced diphosphopyridine nucleotide (DPNH)–cytochrome *c* region was determined by using ferricyanide as the electron acceptor as recommended by Copenhaver & Lardy (1952). Phosphorylation coupled to cytochrome oxidase was studied by the method of Lehninger, ul Hassan & Sudduth (1954). In these experiments the mitochondria were pretreated with 0·075M-sucrose.

Mouse-brain and liver mitochondria were isolated according to Brody & Bain (1952) and Schneider (1948) respectively.

Experiments in vivo. Mice were injected with 50 μg. of crude venom or 75 μg. of heated venom. These doses were equivalent in so far as they were lethal in the same period of time (2·5 hr.). The animals injected with crude venom were killed after 15 min. since it had been observed that after longer intervals the phosphorylation process was completely inhibited. Mice injected with heated venom (phospholipase A) were killed after 30 min. Oxidative phosphorylations located in the regions of (*a*) DPNH–cytochrome *c* and (*b*) cytochrome *c*–oxygen were determined as in the experiments *in vitro*.

RESULTS

Effect of added heated cobra venom (phospholipase A) on phosphorylation in different parts of the respiratory chain. The esterification of inorganic phosphate in two parts of the respiratory chain was studied in mouse-brain and liver mitochondrial preparations. The system in which sodium pyruvate was used as substrate and potassium ferricyanide as the electron acceptor provided a measure of adenosine triphosphate (ATP) synthesis occurring in the region of DPNH–cytochrome *c* (Copenhaver & Lardy, 1952). ATP synthesized during the oxidation of cytochrome *c* was studied by the aerobic oxidation of ferrocytochrome *c* as recommended by Lehninger *et al.* (1954). The latter compound was produced *in situ* by the non-enzymic reduction of ferricytochrome *c* by ascorbate.

The results in Table 1 show the effect of varying the amounts of added heated cobra venom on

Table 1. *Effect of added heated cobra venom on oxidative phosphorylation in mouse-brain and liver mitochondria with ferricyanide as electron acceptor*

The system contained 15 mM-sodium glycylglycine, pH 7·4, 0·01 M-NaF, 0·01 M-Na_2HPO_4–NaH_2PO_4 buffer, pH 7·4, 0·03 M-glucose, 0·15 M-KCl, 8 mM-$MgSO_4$, 2·5 mM-ATP, 13 mM-sodium pyruvate, 2 mM-sodium fumarate, 30 units of hexokinase, 200 equivalent mg. of brain mitochondria or 300 equivalent mg. of liver mitochondria (mitochondria obtained from mg. wet weight of tissue), 17 mM-$NaHCO_3$ and 17 mM-$K_3Fe(CN)_6$ in a total volume of 3·0 ml. Temp., 30°; gas phase, $N_2 + CO_2$ (95:5); period of incubation, 20 min. Venom was tipped from the side arm after equilibration for 5 min.

Venom added (μg./vessel)	Brain			Liver		
	ΔP (μmoles)	$\Delta K_3Fe(CN)_6$ (μmoles)	$P:2K_3Fe(CN)_6$	ΔP (μmoles)	$\Delta K_3Fe(CN)_6$ (μmoles)	$P:2K_3Fe(CN)_6$
—	11·7	13·7	1·8	16·2	19·0	1·7
1	13·6	15·7	1·8	19·2	23·1	1·7
5	11·1	14·8	1·5	15·5	22·2	1·4
20	7·7	14·0	1·1	11·9	19·9	1·2
50	2·0	12·4	0·3	13·5	17·9	0·4
—	11·4	13·4	1·7	18·3	21·3	1·7
1	14·0	16·5	1·7	20·2	23·9	1·7
5	10·3	14·7	1·4	15·7	22·4	1·4
20	7·3	13·2	1·1	10·8	19·6	1·1
50	2·1	10·2	0·4	2·0	17·6	0·1

Table 2. *Effect of added heated cobra venom on oxidative phosphorylation in mouse-brain and liver mitochondria with ascorbate as substrate*

The system contained 5 mM-Na_2HPO_4–NaH_2PO_4 buffer, pH 7·4, 5 mM-$MgSO_4$, 1 mM-sodium ethylenediaminetetra-acetate, 5 mM-ATP, 0·01 M-NaF, 0·2 mM-cytochrome *c*, 0·05 M-sodium ascorbate, 0·03 M-glucose, 30 units of hexokinase and 200 equivalent mg. of brain mitochondria or 300 equivalent mg. liver mitochondria (mitochondria obtained from mg. wet weight of tissue) in a total volume of 3·0 ml. Temp., 30°; gas phase, air; period of incubation, 20 min. The centre well contained 0·2 ml. of 5 N-NaOH. Venom was tipped from the side arm after equilibration for 5 min.

Venom added (μg./vessel)	Brain			Liver		
	ΔP (μmoles)	ΔO (μg.atoms)	P:O	ΔP (μmoles)	ΔO (μg.atoms)	P:O
—	3·2	3·93	0·8	5·3	7·59	0·7
1	2·4	4·02	0·6	4·1	8·21	0·5
5	1·1	3·66	0·3	2·1	6·80	0·3
20	—	4·02	Nil	Nil	7·86	Nil
50	—	4·02	Nil	Nil	6·70	Nil
—	5·5	6·96	0·8	6·2	6·88	0·9
1	4·0	6·76	0·6	6·1	6·60	0·8
5	2·1	6·86	0·3	3·2	6·60	0·5
20	Nil	5·91	Nil	0·6	6·51	0·1
50	Nil	5·77	Nil	Nil	6·06	Nil

the reduction of ferricyanide and on the P:O [$P:2K_3Fe(CN)_6$] ratio. It is seen that 1–5μg. of venom produced a 20% increase in the reduction of ferricyanide by brain and liver mitochondria. Addition of larger quantities of venom (50μg.) only slightly inhibited the reduction of ferricyanide. The phosphorylating system was more susceptible to the action of heated venom. Thus the $P:2K_3Fe(CN)_6$ ratio in the absence of heated venom was generally within the range 1·5–1·9 [values which are in agreement with the results of others (Copenhaver & Lardy, 1952; Pressman, 1955)]. Amounts (5 and 20μg.) of venom depressed the $P:2K_3Fe(CN)_6$ ratio to 1·4 and 1·1 respectively, whereas 50μg. of venom produced almost complete uncoupling in this system.

The results show that in presence of small quantities of venom the uncoupling of phosphorylation is accompanied by stimulation of the oxidative process. Earlier experiments had shown that minute quantities of phospholipase A from cobra venom brought about a pronounced increase in the swelling properties of the mitochondria. It is therefore likely that the observed increase in the rate of electron transport is due to changes in the permeability of the mitochondria, which make the substrate, adenosine diphosphate and orthophosphate more easily accessible to the active centres in the mitochondria.

The results given in Table 2 show the effect of 1–50μg. of heated venom on the respiration and P:O ratio of mitochondria in the presence of cyto-

chrome *c* and sodium ascorbate. These quantities of venom produced only a slight inhibition on the respiratory activity, but the phosphorylation was considerably inactivated. Thus 5μg. of heated venom reduced the P:O ratio from 0·8 to 0·3, and 20μg. caused complete uncoupling. On comparing the results in Tables 1 and 2 it is apparent that the phosphorylation coupled to the oxidation of cytochrome *c* was more susceptible to the action of heated cobra venom than the phosphorylation occurring in the DPNH–cytochrome *c* part of the respiratory chain. Thus 20μg. of heated venom, which completely uncoupled the phosphorylation in the cytochrome *c*–oxygen region, inhibited the phosphorylation in the DPNH–cytochrome *c* part of the chain by only 40%.

Phosphorylations in different parts of the respiratory chain in mitochondria of animals injected with cobra venom. The results of experiments designed to explore the manner in which cobra venom affected the various phosphorylations accompanying respiration in the injected animals are given in Tables 3 and 4. Table 3 shows that the reduction of ferricyanide increased by about 20% in animals injected with crude venom or heated venom. Under these conditions the P:2$K_3Fe(CN)_6$ ratio was lowered from 1·8 to 1·4 in both instances, indicating that the phosphorylation process was inhibited by 20%. Injections of the same quantities of crude and heated venom produced 25% increase in the oxidation of cytochrome *c* but completely inhibited the phosphorylation in the region (Table 4).

On comparing the results given in Tables 3 and 4, it is seen that quantities of venom, which partially inhibit the phosphorylations in the region of DPNH–cytochrome *c*, completely uncoupled the terminal phosphorylation. There is thus complete agreement between the findings of experiments *in vitro* in which heated cobra venom (phospholipase A) was added to the isolated mitochondria and the findings of experiments with mitochondria of animals injected with the toxic material. These experiments demonstrate that the phosphoryl-

Table 3. *Oxidative phosphorylation with ferricyanide as electron acceptor in mitochondria of animals injected with cobra venom*

Conditions are as in Table 1. The results in each experiment represent values obtained from three different groups of animals.

Form of cobra venom injected	Venom injected (μg.)	Time of killing after injection (min.)	Brain			Liver		
			ΔP (μmoles)	$\Delta K_3Fe(CN)_6$ (μmoles)	P:2$K_3Fe(CN)_6$	ΔP (μmoles)	$\Delta K_3Fe(CN)_6$ (μmoles)	P:2$K_3Fe(CN)_6$
None	—	—	10·5	12·4	1·7	13·8	15·3	1·8
			11·1	12·0	1·8	15·4	18·6	1·7
			15·8	20·6	1·5	14·9	18·1	1·6
Crude	50	15	12·2	17·5	1·4	13·4	20·6	1·3
			13·0	18·4	1·3	15·0	19·8	1·5
			11·0	16·6	1·3	14·5	21·8	1·3
Heated	75	30	12·2	17·5	1·4	13·4	20·6	1·3
			13·0	18·4	1·3	15·0	19·8	1·5
			11·0	16·6	1·3	14·5	21·8	1·3

Table 4. *Oxidative phosphorylation with ascorbate as substrate in mitochondria of animals injected with cobra venom*

Conditions are as in Table 2. The results in each experiment represent values obtained from three different groups of animals.

Form of cobra venom injected	Venom injected (μg.)	Time of killing after injection (min.)	Brain			Liver		
			ΔP (μmoles)	ΔO (μg.atoms)	P:O	ΔP (μmoles)	ΔO (μg.atoms)	P:O
None	—	—	3·8	4·20	0·9	5·2	5·53	0·9
			4·5	4·65	0·9	5·8	6·78	0·9
			3·5	4·40	0·8	5·9	6·11	0·7
Crude	50	15	Nil	5·60	Nil	Nil	8·21	Nil
			Nil	5·57	Nil	Nil	8·33	Nil
			Nil	5·51	Nil	Nil	7·81	Nil
Heated	75	30	Nil	6·16	Nil	Nil	9·48	Nil
			Nil	4·81	Nil	Nil	7·40	Nil
			Nil	5·70	Nil	Nil	7·86	Nil

Table 5. *Effect of cobra venom on phosphorylations in different regions of respiratory chain*

Heated venom added (μg.)	Heated venom injected (μg.)	P:O ratios					
		DPNH–O_2		DPNH–cytochrome *c*		Cytochrome *c*–O_2	
		Brain	Liver	Brain	Liver	Brain	Liver
Nil	Nil	2·7	2·7	1·8	1·7	0·8	0·7
20	Nil	1·3	1·3	1·1	1·2	Nil	Nil
Nil	75	1·5	1·5	1·4	1·3	Nil	Nil

ation associated with the oxidation of cytochrome *c* in mitochondria is preferentially inactivated by phospholipase A.

The mitochondria employed for studying oxidative phosphorylation coupled to cytochrome oxidase were pretreated with hypo-osmotic sucrose as recommended by Lehninger *et al.* (1954). It was therefore necessary to know whether the preferential inhibition of phosphorylation in this part of the chain was due to the partial damage caused to the mitochondria by the pretreatment. The results of experiments in which the effects produced by cobra venom on the phosphorylation in the whole respiratory chain were compared with the changes brought about in the phosphorylations occurring in the different regions are summarized in Table 5.

The mitochondria employed for the determination of P:O ratio in the whole respiratory chain as well as in the region of DPNH–cytochrome *c* were prepared under standard conditions (0·25M-sucrose). Table 5 shows that under conditions in which the P:O ratio in the whole chain was partially lowered the amount of inorganic phosphate esterified could be accounted for entirely by the phosphorylations occurring in the region of DPNH–cytochrome *c*. It may therefore be concluded that no phosphorylation occurred in the region of cytochrome *c*–oxygen. Thus the greater susceptibility of the phosphorylation in the region of cytochrome *c*–oxygen to the action of cobra venom could not be due to the damage caused to the mitochondria by the pretreatment with hypo-osmotic sucrose.

DISCUSSION

The results obtained with brain and liver mitochondria have demonstrated that the three phosphorylations linked to the electron-transport chain are not equally susceptible to the destructive action of cobra venom. The enzyme complex concerned with the esterification of inorganic phosphate in the region of cytochrome *c*–oxygen portion of the electron-transport chain is considerably more susceptible to inactivation by the venom. This is apparent from the results of experiments *in vitro*, in which heated cobra venom (phospholipase A) was added to the separated mitochondria, as well as the experiments with mitochondria prepared from animals injected with cobra venom. It is interesting to note that studies on the effect of phospholipase A on the respiration of mitochondria have also shown that cytochrome oxidase is more readily inactivated than some of the other respiratory enzyme systems (Aravindakshan, 1959).

Considerable evidence, including studies with crystalline phospholipase A, indicates that the inhibitory effects produced by cobra venom on oxidative phosphorylation in the mitochondria are due to the destruction of mitochondrial phospholipids by the phospholipase A (Aravindakshan & Braganca, 1961). Mitochondria are rich in phospholipids, and numerous studies have established the importance of phospholipids both in electron transport and in the accompanying phosphorylations (Green, 1959; Schneider, 1959). Recent studies by Green and associates (see Green & Lester, 1959) have resulted in the separation of several different lipoproteins which have been shown to be essential components of the respiratory chain. The nature of the phospholipids concerned with the various phosphorylation steps is less clear. The preferential inactivation produced by phospholipase A on the phosphorylation occurring in the cytochrome *c*–oxygen region could point to differences in the composition of phospholipids essential for the phosphorylation in the different parts of the respiratory chain.

Results obtained with animals injected with phospholipase A (heated venom) are of particular interest. Previous studies have shown that heated cobra venom when added in sufficient quantities to mitochondria from brain or liver can completely uncouple oxidative phosphorylation. Injections of heated venom (phospholipase A) have, however, been found not to lower the P:O ratio to a value less than 1·5. Greater uncoupling effect could not be observed even in animals injected with larger quantities of heated venom or in animals killed after longer intervals (Aravindakshan & Braganca, 1959). In the light of evidence described in this paper, a possible explanation for the above observations may be that in the intact animal the phospholipids concerned with the phosphorylation in the DPNH–cytochrome *c* region are considerably resistant to the action of phospholipase A.

SUMMARY

1. Experiments with brain and liver mitochondria have demonstrated that the three phosphorylations linked to the electron-transport chain are not equally susceptible to the uncoupling action of cobra venom.

2. The phosphorylation in the region of cytochrome oxidase is preferentially inactivated by heated venom. These observations have been made in direct experiments *in vitro* and with mitochondria obtained from animals injected with venom.

3. Evidence obtained with mitochondria of animals injected with heated venom (phospholipase A) suggests that the phospholipids concerned with phosphorylations in the reduced diphosphopyridine nucleotide–cytochrome *c* region are considerably resistant to the action of phospholipase A.

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Studies on Phospholipid Structures in Mitochondria of Animals Injected with Cobra Venom or Phospholipase A

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Considerable evidence has been obtained that venoms containing phospholipase A bring about inactivation of respiratory activity and uncouple oxidative phosphorylation when added to isolated tissue preparations (Braganca & Quastel, 1952, 1953; Nygaard, 1953*a*, *b*; Nygaard & Sumner, 1953; Petrushka, Quastel & Scholefield, 1957). It has been suggested that these effects are produced through the destructive action of phospholipase A on mitochondrial phospholipids. Nygaard (1953*a*) has shown that the inactivation of succinic oxidase by phospholipase A from *Crotalus terrificus* venom is dependent on the extent of phospholipid hydrolysed. Results of investigations on the changes produced on oxidative phosphorylation in the mitochondria of animals injected with cobra venom have shown remarkable agreement with findings of experiments *in vitro*. The enzyme systems concerned with esterification of inorganic phosphate appear to be particularly sensitive to the action of cobra venom (Aravindakshan & Braganca, 1959). The present investigation was undertaken to study in greater detail the mechanism underlying the uncoupling action of cobra venom on oxidative phosphorylation in mitochondria. Data from studies on submitochondrial particles as well as on the swelling properties of mitochondria show that cobra venom brings about pronounced changes in mitochondrial structure. These effects have been observed *in vitro* as well as in animals injected with cobra venom or with crystalline phospholipase A.

Several reports have appeared on the uncoupling effect of lysolecithin on oxidative phosphorylation

(Habermann, 1954; Witter, Morrison & Shepardson, 1957). Lysolecithin is also known to enhance the swelling of mitochondria (Witter & Cottone, 1956). Quantitative studies designed to explore to what extent the changes produced by cobra venom may be attributed to the effect of lysolecithin indicate that the lysolecithin formed from the quantities of venom added is too small to be effective. It appears from these findings that the destructive effects of cobra venom on mitochondrial metabolism arise primarily through direct hydrolysis of essential mitochondrial phospholipids by the phospholipase A.

EXPERIMENTAL

Rat-liver submitochondrial fragments were prepared according to Cooper & Lehninger (1956). They were characterized by determination of the P:O ratio and by some of the properties in which they differ from intact mitochondria as shown in Table 1. It is seen that Ca^{2+} ions, which uncouple oxidative phosphorylation and also activate the adenosine-triphosphatase activity in fresh mitochondria, did not affect these properties in the submitochondrial fragments. Mg^{2+} ions, which neither inhibit the P:O ratio nor activate the adenosine triphosphatase in fresh mitochondria, completely inhibited phosphorylations as well as stimulated the adenosine triphosphatase in the fragments. 2:4-Dinitrophenol inhibited phosphorylations and also activated the adenosine triphosphatase in the fragments, as it does in the fresh intact mitochondria. These results showed that the submitochondrial particles employed in these studies had properties identical with those described by Cooper & Lehninger (1956) for the original preparation of these fragments.

Table 1. *Properties of submitochondrial fragments prepared from rat liver*

(*a*) *Oxidative phosphorylation*

The system contained 0·01 M-DL-sodium β-hydroxybutyrate, 2·4 mM-adenosinetriphosphate (ATP), 5 mM-Na_2HPO_4–NaH_2PO_4 buffer, pH 6·0, 0·1 mM-$MgSO_4$, 0·01 M-NaF, submitochondrial fragments containing 2·5–3·0 mg. of protein, 0·03 M-glucose, and 30 units of hexokinase (Berger, Slein, Colowick & Cori, 1946) in a total volume of 3·0 ml. The centre well contained 0·2 ml. of 5 N-NaOH. Gas phase, air; temp., 30°; period of incubation, 30 min.

(*b*) *Adenosine-triphosphatase activity*

The system contained 4 mM-ATP, 0·02 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7·4, and submitochondrial fragments containing 0·4–0·5 mg. of protein in a total volume of 1·0 ml. Temp., 30°; period of incubation, 10 min.

Substance added	Concn. of the substance (mM)	P:O ratio	Relative adenosine-triphosphatase activity (μmoles of P liberated)
None	—	2·1	0·33
2:4-Dinitrophenol	0·05	0·2	1·12
Ca^{2+} ions	3	2·2	0·35
Mg^{2+} ions	3	Nil	1·71

Heated cobra (*Naja naja*) venom was prepared by heating solutions of crude venom at 100° for 15 min. as described by Braganca & Quastel (1953). The amount of heated venom employed in experiments is expressed in terms of the amount of crude venom from which it was obtained. Crystalline phospholipase A was prepared from crude cobra venom according to Suzuki, Iwanaga & Kawachi (1958).

Mouse-liver mitochondria used for studying the swelling properties were prepared according to Tapley (1956). For other experiments mouse-brain mitochondria were prepared by the method of Brody & Bain (1952) and liver mitochondria were isolated according to Schneider (1948). The amount of mitochondria used in experiments is expressed as equivalent mg. (the wet weight of the tissue in mg., from which the mitochondria are obtained).

Lysolecithin was prepared by a modification of the procedure employed by Hanahan, Rodbell & Turner (1954). In this preparation 5 g. of commercial egg lecithin was dissolved in 500 ml. of peroxide-free ethyl ether and mixed with 5 ml. of an aqueous solution of 30 mg. of heated cobra venom. The precipitate so formed was removed after 3 hr. by centrifuging and dissolved in the minimum amount of chloroform. The turbid solution obtained was again centrifuged at 15 000 *g* for 30 min. and the residue discarded. To the clear solution 6 vol. of ether was added, and the precipitate was recovered and washed with ether. This procedure was repeated four times. The product obtained was characterized by analysis for phosphorus, nitrogen and fatty acid content. Nitrogen was determined by the micro-Kjeldahl procedure of Johnson (1941). Phosphorus was determined according to King (1930). The amount of fatty acid was estimated by the saponification method (Vogel, 1951). The results given below show that the analytical results obtained are in close agreement with theoretical values and the data reported by Hanahan *et al.* (1954). Theoretical values (%): N, 2·76; P, 6·07; fatty acid, 49·7. Observed values (%): N, 2·80; P, 5·91; fatty acid, 47·1. The absence of any contamination of lysolecithin by phospholipase was demonstrated by the Warburg manometric method described by Braganca & Quastel (1953). The results showed that 10 mg. of lysolecithin produced no carbon dioxide within a period of 2 hr. The minimum lethal dose (MLD) is defined as the minimum dose of the venom preparation expressed in terms of protein content which when injected subcutaneously into 2–6-months-old mice (weighing 20–25 g.) killed all the mice within 18 hr.

RESULTS

Effect of cobra venom on oxidative phosphorylation in submitochondrial fragments

Recent work on the mechanism of oxidative phosphorylation has shown that uncoupling agents in general may be classified into two types: (1) those which uncouple by direct interference with the phosphorylation process; (2) those which inhibit the phosphorylation process indirectly through their action on the structural organization of the mitochondria. Uncoupling agents of the first type have been found effective both in intact mitochondria as well as in submitochondrial fragments

capable of oxidative phosphorylation. Agents belonging to the second type produced uncoupling only in intact mitochondria (Lehninger, Wadkins, Cooper, Devlin & Gamble, 1958). Since the structural organization of mitochondria is already disrupted during the preparation of the submitochondrial fragments, uncoupling agents of this class do not produce any effect in this system. These preparations therefore serve as a tool for studying the mechanism of the uncoupling action of any agent.

In the present studies submitochondrial fragments were prepared according to Cooper & Lehninger (1956) and characterized as described above. Studies with intact mitochondria have shown that the uncoupling action of cobra venom on the phosphorylation process is dependent on the relative proportion of venom added to mitochondrial protein. The ratio of venom to mitochondrial protein in the experiments with submitochondrial fragments described in Table 2 is the same as the ratio of venom to mitochondrial protein reported in the earlier findings with intact mitochondria (Aravindakshan & Braganca, 1959). Table 2 shows that 15μg. of heated venom had no effect on the respiration or the P:O ratio in this system. This amount of venom had been found to produce 30% inhibition on the phosphorylation process in the system containing intact mitochondria. When 100μg. of heated venom was employed both the oxygen absorption and the uptake of inorganic phosphate were equally inhibited. It is apparent from these results that cobra venom does not directly inhibit the phosphorylating enzymes.

Effect of cobra venom on the swelling properties of liver mitochondria

Studies on phosphorylation accompanying oxidation, carried out with intact mitochondria and also with submitochondrial particles, suggest that venom affects this process indirectly through its action on the mitochondrial structure. Direct evidence to show that cobra venom produces structural changes in the mitochondria was provided by a study of the effects of the venom on the spontaneous swelling of liver mitochondria. Mouse-liver mitochondria were prepared in 0·44 M-sucrose and were suspended in the test system containing 0·3 M-sucrose. It has been shown by Tapley (1956) that in this concentration of sucrose the mitochondria swell at a moderate rate, and the system is therefore convenient for the study of the effects of agents which either enhance or prevent swelling. Tedeschi & Harris (1955) have demonstrated that $E_{520\,m\mu}$ of suspensions of rat-liver mitochondria in sucrose solutions is inversely related to the mitochondrial volume. In the present studies the decrease in $E_{520\,m\mu}$ is taken as a measure of the extent of swelling.

Fig. 1 shows the changes produced in the swelling properties by heated cobra venom and crystalline phospholipase A. Addition of 0·1μg. of heated venom produced 16% increase in the extent of

Table 2. *Effect of heated cobra venom on oxidative phosphorylation in sub-mitochondrial fragments*

Conditions are as in Table 1. Heated venom was tipped from the side arm after equilibration for 5 min.

Tissue (mg. of protein/ vessel)	Venom (μg./vessel)	ΔP (μmoles)	ΔO (μg.atoms)	P:O
2·74	—	7·5	3·57	2·1
	0·15	6·8	3·39	2·0
	0·75	6·9	3·30	2·1
	3·0	6·8	3·39	2·0
	7·5	6·2	3·12	2·0
2·71	—	7·2	3·12	2·3
	0·15	6·6	2·86	2·3
	0·75	7·2	3·03	2·4
	3·0	7·3	3·20	2·3
	7·5	7·2	3·03	2·4
	15·0	7·5	3·12	2·4

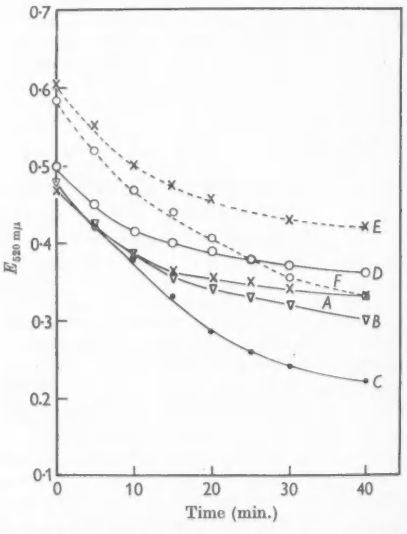

Fig. 1. Effect of addition of phospholipase A and heated venom on the swelling of mouse-liver mitochondria. The test system contained 40 equivalent mg. of mitochondria (mitochondria obtained from 40 mg. wet weight of tissue) in 0·3 M-sucrose, 0·02 M-tris buffer, pH 7·4, in a total volume of 3·0 ml. Temp., 25°. (i) *A*, Control; *B*, 0·1μg. of heated venom; *C*, 0·5μg. of heated venom; *D*, 0·5μg. of heated venom + 1·0 mg. of egg lecithin. (ii) *E*, Control; *F*, 0·026μg. of crystalline phospholipase A.

swelling in 40 min. (curve *B*) and 0·026μg. of phospholipase A increased the swelling by 45% in the same period (curves *E* and *F*). Higher concentration of heated venom produced greater effects (curve *C*). Increased swelling of mitochondria has been observed on addition of venom from *Agkistridon piscivorus* (Petrushka *et al.* 1957). These findings indicate that changes produced by cobra venom on the swelling of mitochondria are brought about through the action of phospholipase A on phospholipids of mitochondrial membrane. It was of interest to find that 1·0 mg. of egg lecithin completely reversed the increase in swelling brought about by 0·5μg. of heated venom (curves *C* and *D*). This quantity of lecithin alone had no effect on the swelling process. The protective effect of lecithin on the uncoupling of oxidative phosphorylation by *A. piscivorus* venom has been demonstrated by Petrushka *et al.* (1957). Presumably lecithin is able to compete with the phospholipids of the mitochondria for the added phospholipase.

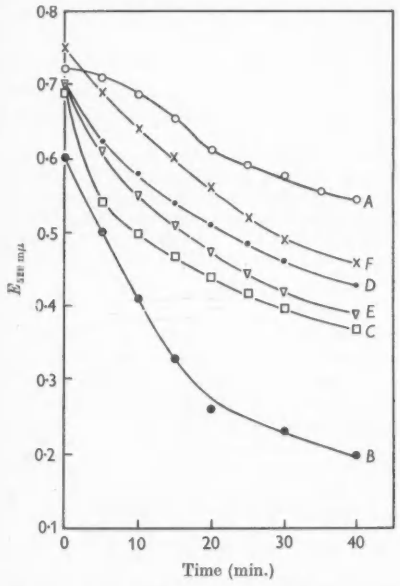

Fig. 2. Effect of injected cobra venom and phospholipase A on the swelling properties of liver mitochondria. Assay system was as in Fig. 1. *A*, control; *B*, mice injected with 50μg. of crude venom and killed after 30 min.; *C*, mice injected with 75μg. of heated venom and killed after 30 min.; *D*, mice injected with 50μg. of crude venom and killed after 10 min.; *E*, mice injected with 9μg. of crystalline phospholipase A and killed after 30 min.; *F*, mice injected with 22·5μg. (MLD) of crude venom and killed after 30 min. The results are representative of at least six experiments.

In order to explore the changes produced by cobra venom in the intact animal, the rate of swelling of mitochondria isolated from mice injected with cobra venom was investigated. In the first series of experiments mice were injected with 50μg. of crude venom, 75μg. of heated venom or 9μg. of crystalline phospholipase A. These doses were lethal in 2·5 hr. The mice were then killed after 10 or 30 min. As seen in Fig. 2, mitochondria of the experimental animals (curves *B* and *C*) swelled at a faster rate compared with those of control animals (curve *A*). Changes in mitochondrial swelling could be detected within an interval of 10 min. after the animals were injected with crude venom (curve *D*). Injections of crystalline phospholipase produced changes in mitochondria similar to those brought about by crude venom and heated venom, the extent of swelling being 37% higher than in the controls (curve *E*). In another series of experiments animals were injected with a minimum lethal dose of crude venom (this dose was lethal in 8–10 hr.). Mitochondria of such animals killed 30 min. after the injection of the minimum lethal dose were also considerably damaged as shown by the greater extent of swelling (curve *F*) as compared with the controls.

Effect of lysolecithin on oxidative phosphorylation and on the swelling of mitochondria

Since lysolecithin is known to uncouple oxidative phosphorylation and produce swelling of liver mitochondria it was necessary to determine whether the destructive effects produced by phospholipase A on mitochondrial metabolism were due to the lysolecithin produced. This question was explored earlier by Petrushka, Quastel & Scholefield (1959). They observed that phospholipase C from *Clostridium welchii*, which does not give rise to lysolecithin, behaved like phospholipase A in inhibiting mitochondrial respiration, from which they concluded that the destructive effects of phospholipase are not due to the lysolecithin formed. In view of these observations, a quantitative study was carried out to determine to what extent lysolecithin might be implicated in the uncoupling action of cobra venom on oxidative phosphorylation and its effects on the swelling of mitochondria under the experimental conditions employed.

In these experiments 50μg. of heated venom were incubated with brain and liver mitochondria under similar conditions to those employed for the study of oxidative phosphorylation. As reported earlier this quantity of venom produced complete uncoupling of oxidative phosphorylation (Aravindakshan & Braganca, 1959). After incubation for

20 min. the lipids were extracted according to Marinetti, Scaramuzzino & Stotz (1957), and the lysophospholipids estimated by their haemolytic action on a standard system of human erythrocytes (Bernheimer, 1947). The quantities of lysophospholipids are expressed in terms of lysolecithin. These experiments showed that 200 equivalent mg. of brain mitochondria or 300 equivalent mg. of liver mitochondria gave rise to 50–75μg. of lysolecithin. Under similar conditions mitochondria incubated without the venom did not produce detectable amounts of lysophospholipids. Witter & Cottone (1956) also did not detect lysolecithin in fresh liver mitochondria.

Effects of graded amounts of lysolecithin on the P:O ratio and on the swelling properties of mitochondria are shown in Table 4 and Fig. 3 respectively. It is evident from Table 4 that 0·25 and 0·5 mg. of lysolecithin increased the respiration, whereas 2·0 mg. of lysolecithin inhibited the oxygen uptake by about 40%. There was no effect on oxidative phosphorylation by 0·25 mg. of lysolecithin. Quantities higher than this (0·5–2·0 mg.) depressed the P:O ratio progressively to nil. It is therefore clear that lysolecithin in amounts which are produced by the action of venom on the mitochondria does not have any effe·t on the respiration or on the phosphorylation process.

Fig. 3 shows the effect of lysolecithin on the swelling of mouse-liver mitochondria in 0·3 M-sucrose. There was no effect on the swelling by 1·0μg. of lysolecithin, whereas 50μg. enhanced swelling by 78%. An estimate of the quantity of lysolecithin that could be produced by the action of heated venom on the amounts of mitochondria employed in these experiments may be extrapolated from the data given in Table 3. It is evident that no more than 1μg. of lysolecithin could be produced under these conditions. Thus it is apparent from these studies that lysolecithin could

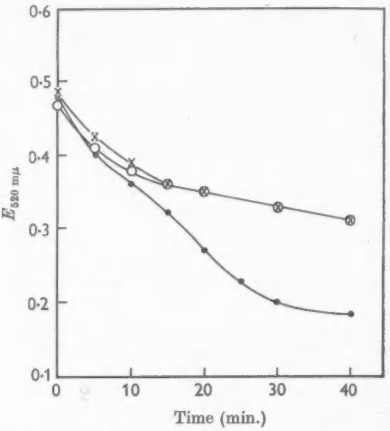

Fig. 3. Effect of lysolecithin on the swelling properties of mouse-liver mitochondria. Assay system was as in Fig. 1. ×, Control; ○, 1·0μg. of lysolecithin; ●, 50μg. of lysolecithin.

Table 3. *Liberation of lysophospholipids from mouse-brain and liver mitochondria by heated cobra venom*

Brain mitochondria (200 equivalent mg.) or 300 equivalent mg. of liver mitochondria (mitochondria obtained from mg. wet weight of tissue) suspended in 0·15 M-KCl in a total volume of 3·0 ml. were incubated with venom at 30° for 20 min. $E_{540\ m\mu}$ was taken as a measure of haemolysis.

Venom added (μg.)	Lysolecithin formed (μg.)	
	Brain	Liver
Nil	Nil	Nil
50	70·6	63·0
50	61·6	53·2
50	68·4	56·6

Table 4. *Effect of added lysolecithin on oxidative phosphorylation in mouse-brain and liver mitochondria*

The system contained 15 mM-sodium glycylglycine, pH 7·4, 0·01 M-NaF, 0·01 M-Na_2HPO_4–NaH_2PO_4 buffer, pH 7·4, 0·03 M-glucose, 0·15 M-KCl, 8 mM-$MgSO_4$, 2·5 mM-ATP, 13 mM-sodium pyruvate, 2 mM-sodium fumarate, 30 units of hexokinase and 200 equivalent mg. of brain mitochondria or 300 equivalent mg. of liver mitochondria (mitochondria obtained from mg. wet weight of tissue) in a total volume of 3·0 ml. The centre well contained 0·2 ml. of 5 N-NaOH. Temp., 30°; gas phase, air; period of incubation, 20 min. Lysolecithin was tipped from the side arm after equilibration for 5 min.

Lysolecithin (mg./vessel)	Brain			Liver		
	ΔP (μmoles)	ΔO (μg.atoms)	P:O	ΔP (μmoles)	ΔO (μg.atoms)	P:O
—	12·2	4·37	2·8	14·5	5·80	2·5
0·25	12·3	4·64	2·8	18·0	6·96	2·6
0·50	11·1	5·36	2·1	12·2	6·42	1·9
1·00	8·0	4·01	1·5	5·5	4·60	1·2
1·50	2·6	2·41	1·1	1·9	2·77	0·7
2·00	1·0	2·63	0·3	Nil	2·41	Nil

Table 5. *Effect of lysolecithin on oxidative phosphorylation in rat-liver submitochondrial fragments*

The system was as in Table 1.

Tissue (mg. of protein/ vessel)	Lysolecithin added (μg.)	ΔP (μmoles)	ΔO (μg.atoms)	P:O
2·62	—	4·9	2·32	2·1
	75	4·2	2·23	1·9
	150	5·0	2·50	2·0
	225	5·2	2·59	2·0
	300	5·1	2·41	2·1
2·67	—	6·4	3·21	2·0
	75	6·2	2·94	2·1
	150	6·9	3·20	2·1
	225	6·9	3·30	2·1
	300	6·1	3·03	2·0

not be responsible for the various effects produced by cobra venom on mitochondrial functions.

To study the mechanism of the uncoupling action of lysolecithin, the effect of this compound on oxidative phosphorylation in submitochondrial fragments was investigated. Table 5 shows that 300μg. of lysolecithin had no effect on the respiration or on the P:O ratio in this system. Table 4 shows that this concentration of lysolecithin in relation to the mitochondrial protein produced almost complete uncoupling in intact mitochondria. These findings demonstrate that lysolecithin has no effect on the phosphorylation process, and, like venom, uncouples oxidative phosphorylation in intact mitochondria through its action on the mitochondrial structure.

DISCUSSION

Studies described in this paper have shown that although both heated venom (phospholipase A) and lysolecithin can uncouple oxidative phosphorylation in fresh mitochondria, they have no action on the phosphorylation accompanying respiration in submitochondrial particles. This clearly shows that they do not have direct inhibitory action on the phosphorylating enzyme systems. Observation on the effects of heated venom and lysolecithin on the swelling properties of isolated liver mitochondria of normal animals have also demonstrated that addition of minute quantities of these materials can bring about pronounced changes in the mitochondrial membrane. It is satisfying to find complete correlation between these findings and the observations made on the mitochondria of animals injected with cobra venom. Thus the mitochondria of animals injected with venom showed greater susceptibility to swell than those of controls. The cobra toxin apparently damages the mitochondrial structure very rapidly, as changes in the swelling properties of liver mitochondria could be detected within 10 min. after the injection. These findings suggest that changes in the mitochondrial membrane may be among the early biochemical disturbances produced by cobra venom on the mitochondrial metabolism. Experiments *in vitro* have shown that the swelling of mitochondria is produced with smaller quantities of the toxin than those required to produce changes in the P:O ratio, indicating that changes in the permeability probably precede the effects on other mitochondrial functions. Experiments *in vitro* as well as with injected animals show that the effects produced by crystalline phospholipase *A* on mitochondrial metabolism are similar to changes brought about by cobra venom. Minute quantities can uncouple oxidative phosphorylation and also produce considerable increase in the swelling properties of the mitochondria. It is well established that mitochondria are rich in phospholipids, and these are known to play an important role in the phosphorylation process (Green, 1959; Schneider, 1959). It is evident from the reports of Petrushka *et al.* (1957) as well as the present studies that heated venom can hydrolyse mitochondrial phospholipids. These observations therefore support the conclusion that cobra venom disturbs mitochondrial metabolism through the destructive action of phospholipase on mitochondrial phospholipids. The effects observed in the intact animal are presumably mediated by a similar mechanism. Since it has already been demonstrated that the phospholipase A of cobra venom can penetrate the blood–brain barrier and bring about hydrolysis of brain phospholipids (Aravindakshan & Braganca, 1959) it is clear that phospholipase A can inactivate mitochondrial functions in the brain in the intact animal.

The data regarding the uncoupling action of lysolecithin reported here appear to be in disagreement with the results of Witter *et al.* (1957). Although these authors have employed rat-liver tissue, whereas the present findings relate to mouse tissues, the amounts of mitochondria employed in both investigations are of the same order. Several studies (Marples & Thompson, 1958; Saunders, Thomas & Robinson, 1958) have reported that phospholipase is a contaminant of lysolecithin preparations. The sample of lysolecithin employed in the present study was therefore shown by analysis to be free from phospholipase or lecithin. Witter *et al.* (1957) have analysed their sample of lysolecithin for C, H and P. This analysis, however, could not determine the presence of traces of phospholipase. It is possible therefore that the greater effect of lysolecithin on oxidative phosphorylation reported by these authors may have been due to small quantities of venom present as impurity in the sample of lysolecithin.

Quantitative experiments, carried out to determine to what extent the various changes in the mitochondrial properties result from the effects of lysolecithin, have clearly shown that the quantity of lysolecithin formed from venom under the experimental conditions could not possibly account for the various effects observed. These studies do not exclude the possibility that lysolecithin produced from venom could be more localized on the surface of the mitochondria and as such may have greater destructive effects on mitochondrial metabolism than added lysolecithin. The studies described have, however, shown that lecithin reverses the action of venom on the swelling of mitochondria *in vitro*, presumably owing to a competition of lecithin with mitochondrial phospholipids for the phospholipase. Lecithin also reverses the uncoupling effect of venom on oxidative phosphorylation (Petrushka *et al.* 1957). These observations are in favour of the view that the various effects are brought about by the direct action of venom phospholipase on the phospholipids of the mitochondria. Attempts to reverse, with lecithin, the effect of cobra venom on the swelling of mitochondria in the injected animals were, however, not successful. It is of interest that Nygaard, Dianzani & Bahr (1954) have observed that lysolecithin and phospholipase A produce qualitatively different types of changes in the morphology of the mitochondria as detected by electron microscopy.

SUMMARY

1. Heated cobra venom did not uncouple oxidative phosphorylation in submitochondrial fragments prepared by digitonin extraction. These experiments suggest that venom does not directly inhibit the phosphorylating enzymes.

2. Addition of small quantities of crystalline phospholipase A enhanced spontaneous swelling of fresh mitochondria. The effect could be reversed by lecithin. Mitochondria of animals injected with phospholipase also showed greater swelling than the controls.

3. Evidence presented demonstrates that the various effects produced by cobra venom on mitochondrial metabolism result from hydrolysis of mitochondrial phospholipids by the phospholipase. The quantity of lysolecithin produced could not account for the various effects of cobra venom on mitochondrial metabolism.

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Studies on Vitamin E

6. THE DISTRIBUTION OF VITAMIN E IN THE RAT AND THE EFFECT OF α-TOCOPHEROL AND DIETARY SELENIUM ON UBIQUINONE AND UBICHROMENOL IN TISSUES*

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Although vitamin E deficiency and the phenomena associated with it in the rat have been studied continuously for many years, the nature of the role of vitamin E still remains obscure. Some workers consider that its role in animals may be simply defined by equating α-tocopherol with an intracellular antioxidant whose presence is necessary to prevent the accumulation of substances that would otherwise lead to peroxidation of tissue lipids and thus would interfere with normal metabolism. Other workers believe that the vitamin may play a more specific role, perhaps in connexion with electron transport or intracellular phosphorylation. It is difficult, indeed, to separate the first function (that of antioxidant) from any other that may exist.

One of the more interesting features of vitamin E deficiency is the variety of symptoms that it produces in different animals, and sometimes, by a slight change in experimental conditions, in the same animal. It seemed, at the outset of this work, that more information than was hitherto available about the distribution of tocopherol in the tissues of animals was desirable. Even our knowledge about the distribution of vitamin E, under varying nutritional conditions, in such a well-studied animal as the rat is limited. For example, examination of the literature failed to reveal any information about the vitamin E content of such a significant organ as normal rat uterus, and practically nothing appeared to be known about the actual concentrations of tocopherol in vitamin E-deficient tissues. Methods for the analysis of such tissues are now available (Edwin, Diplock, Bunyan & Green, 1960; Diplock, Green, Edwin & Bunyan, 1960*b*) and have extended the range of determination to encompass all the types of biological material so far encountered.

If a single thread can be said to run through the skein of observations on the effects of vitamin E deficiency, it would seem to be that there is some relation between the vitamin and cellular oxidation processes. The recent discovery of the role of ubiquinone (coenzyme Q) in electron transport (Crane, Hatefi, Lester & Widmer, 1957) and the identification of ubichromenol, the cyclized isomer of ubiquinone, by Laidman, Morton, Paterson & Pennock (1960) have provided a new starting point for a consideration of this problem. Fortunately both these substances could be studied in tissues by the same methods as used for tocopherols and, in the present work, the relationships that exist between these groups of lipids in the rat have been investigated in some detail. In view of the interactions between vitamins A and E (the nature of which became clearer as the work progressed), vitamin A analyses were carried out on the extracts wherever possible. Because of the role of selenium in vitamin E-deficiency conditions (Schwarz & Foltz, 1957), some preliminary investigations were made into the influence of dietary selenium on the concentrations of ubiquinone and ubichromenol.

* Part 5: Bunyan, Green, Edwin & Diplock (1960).

EXPERIMENTAL

Animals

Rats of the Norwegian hooded strain, of both sexes, were used. They were removed from the stock colony at about 13 days of age and placed on the appropriate test diet.

Diets

Stock diet (*SD*1). This was used in all tests in which a nutritionally adequate control diet was required: wheat germ (15%), meat and bone meal (5%), dried milk (10%), grass meal (5%), soya-bean meal (2%), dried brewer's yeast (3%), salt (0·53%), arachis oil (5%) and maize meal to 100%. The diet was supplemented with a stabilized vitamin A and D preparation to provide 1000 i.u. of vitamin A and 100 i.u. of vitamin D/lb.

Vitamin E-deficient basal diet (*A*40). This contained casein (Genatosan Ltd., 'low vitamin content'; 25%), sucrose (50%), dried yeast (Marmite Food Extract Co.; 10%), lard (Wiltshire United Dairies; 10%) and McCollum's salt mixture (5%) to provide (g./kg. of diet) the following: NaCl, 2·3; $CaH_4(PO_4)_2$, 7·3; ferric citrate, 1·57; NaH_2PO_4, 4·7; $MgSO_4,7H_2O$, 3·6; K_2HPO_4, 12·9; calcium lactate, 17·5; KI, 0·06; NaF, 0·015; $MnSO_4,4H_2O$, 0·06; Cu_2I_2, 0·005; $K_2SO_4,Al_2(SO_4)_3,H_2O$, 0·005. The diet was supplemented with vitamin A palmitate (40 i.u./g.) and vitamin D_3 (2·5 i.u./g.).

Vitamin E-supplemented basal diet (A40E). This was identical with A40, with the addition of DL-α-tocopheryl acetate (0·1 mg./g.).

Necrogenic diet. This was that described by Green, Edwin, Bunyan & Diplock (1960).

Plan of animal experiments

Vitamin E deficiency. Rats were divided into groups of 10 or 12. Males and females were used in separate but replicate groups, there being 11 groups in all. The four groups of males were put on the test diets thus: groups A and B on diet A40, C on A40E and D on the stock diet SD1. The animals in group B were litter-mate controls for those in group A. The groups were maintained on these diets until they were 6 months old. At 72 hr. before the end of the experiment, and again at 48 hr., the rats in group B were given an oral dose of 5 mg. of DL-α-tocopheryl acetate. After 48 hr., all the animals were killed. A similar experiment was carried out with four groups of female rats (E, F, G and H, the first two groups being randomized from litter mates). In addition, three groups of females were reared to 13 months, thus: groups J and K, of randomized litter mates, on diet A40; group L on diet SD1. Groups F and K were dosed orally with α-tocopheryl acetate 72 and 48 hr. before death, as in the experiment on the males. Animals on the deficient diet A40 showed typical symptoms of vitamin E deficiency. These included depigmentation of the incisors, testicular degeneration in males and brown uterus and failure of gestation in females.

Selenium deficiency. Three experiments were carried out. In the first, weanling rats of both sexes were divided into two groups, M and N. Group M consisted of six males and eight females, which were maintained on the necrogenic diet for 7 weeks; group N, five males and four females, were on the same diet supplemented with selenium (0·05 p.p.m. as sodium selenite). In the second experiment, four groups of 12 rats of mixed sexes were given the following diets respectively: group O, the necrogenic diet; group P, the same diet with added selenium (0·05 p.p.m.); group Q, the diet with added α-tocopheryl acetate (100 p.p.m.); group R, the diet with both additions. These rats were maintained on test for 6 weeks, during which time five of the group O rats died. In the third experiment, two groups of four rats (two of each sex) aged about 3 months were given a modified vitamin E-deficient diet (similar to A40, but with only one-quarter of the vitamin A). Group T were given supplementary selenium (0·1 p.p.m.) for 2 weeks, group S acting as controls.

Analytical methods

At the end of each experiment, the rats were anaesthetized with ether, samples of blood taken from the hearts and the rats killed by cardiac excision. The animals were then dissected at once. The following tissues were taken: heart, liver, kidney, skeletal muscle (hind leg), spleen, brain, adrenal gland, lung, sciatic nerve, uterus or testis and a sample of body fat removed from below the kidneys. The organs were weighed and then the tissues from each group were pooled and stored in polythene bottles at −20° until they could be analysed (usually within a few days, occasionally as long as 4 weeks). Previous studies (Diplock *et al.* 1960*b*) showed that no significant losses of ubiquinone, ubichromenol or vitamin E occurred during storage at low temperatures; nevertheless, to minimize variation, it was always the practice to analyse each tissue in parallel with its various controls on the same day. The adrenals were deep-frozen immediately after removal from the animal. Blood was separated into clot and serum and these were analysed separately.

The analyses of tissues and of diets were by the two-dimensional chromatographic procedure of Diplock *et al.* (1960*b*), vitamin A being measured on the same extracts, as previously described. For analysis, 10–20 g. of tissue was taken. If this amount was not available, the total available quantity was used. For analyses of the diets and dietary ingredients, 50–250 g. of each was extracted exhaustively with ether and the analysis carried out on the ether extract.

The value for $\Delta E^{1\%}_{1\,cm.}$ at 274·5 mμ in the borohydride assay of the quinones was taken as 152 throughout, this figure being that calculated for ubiquinone 45, the main component. Although some tissues may have contained a greater proportion of ubiquinone 50 than others, only slight changes in the results were produced by modifying the ΔE factor to take account of fluctuations in the isoprenologue ratios, and it was not considered that the analytical results would be significantly improved by so doing. Similarly, the same spectrophotometric-conversion factor was used throughout for the determination of ubichromenol, whatever the apparent changes in isoprenologue pattern. The factor for ubichromenol 45 (see Diplock *et al.* 1960*b*) was checked by determining a nearly pure specimen of ubichromenol 50 (kindly given by Professor R. A. Morton and Dr J. F. Pennock), then allowing for the difference in molecular weight. The best value we could obtain was taken as 300 for ubichromenol 50, which gave a factor of 270 for ubichromenol 45, in good agreement with that found previously. In all probability the true factor is somewhat lower than this, but its more accurate assessment must await a further supply of the pure substance.

RESULTS

Analysis of the diets and some dietary ingredients

To compare the intake of vitamin E on the different nutritional regimens, the two key diets, SD1 and A40, were analysed (Table 1). In addition, the ingredients of A40 likely to introduce tocopherol into the diet were analysed individually.

Table 1. *Analysis of diets and dietary components*

n.d., Not detected; tr., trace.

Material	Ubiquinone (μg./g.)	α-Tocopherol (μg./g.)	β-Tocopherol (μg./g.)
Stock diet	1·2	9·5	2·7
Diet A40	0·8	2·4	n.d.
Lard*	n.d.	7·9	n.d.
Casein†	tr.	0·7	0·1
Yeast (dried)‡	9·9	tr.	n.d.
Yeast (dried)§	1·1	0·3	n.d.

* Wilts. United Dairies.
† Genatosan Ltd. 'low vitamin content'.
‡ Marmite Food Extract Co.
§ Distillers Co. Ltd.

It will be observed that the vitamin E-deficient diet contains about a quarter of the tocopherol of the adequate stock diet and that most of this comes from the lard. The total amount of tocopherol in the whole diet A 40 was rather more than could be accounted for by summation of the ingredients, but there is a good deal of uncertainty in the determination of these small quantities: large amounts of material must be extracted and the final measurements are small. The presence of α-tocopherol in the yeast will be discussed elsewhere.

Tissue analyses, analytical precision and the effect of added α-tocopherol on the recovery of ubiquinone and ubichromenol

The results of the various experiments on the distribution of vitamin E in the rat and its effect on other lipid constituents are presented in Tables 2–4, grouped to illustrate sex and age differences. The only tocopherol measurements recorded are for α-tocopherol. Occasionally small amounts of β-tocopherol were observed in the rats on diet SD 1 and these were, in fact, measured; but, since the quantities found did not contribute significantly to the total vitamin E, they have not been included in the tables. Rats on the other diets contained only α-tocopherol. The figures for ubiquinone and ubichromenol refer to the total isoprenologue content in each case. Chromatographic separation (Diplock *et al.* 1960*b*) showed that the tissue extracts of most groups of animals contained mainly ubiquinones 45 and 50. However, the tissues from group L all contained ubiquinones 45, 40 and 35. The ubichromenol pattern corresponded to the quinone pattern (Diplock, Edwin, Green, Bunyan & Marcinkiewicz, 1960*a*).

As already shown by Diplock *et al.* (1960*b*) and confirmed at several points during these experiments, recoveries of ubiquinone, ubichromenol and tocopherol appeared to be essentially quantitative. In Table 5 are recorded the results of four experiments designed to determine the precision of the analytical methods. In view of our findings on the influence of vitamin E on concentrations of ubiquinone and ubichromenol in the tissues, it was essential to examine whether the addition of α-tocopherol to tissues could itself affect the recovery of either ubiquinone or ubichromenol. This was done by adding α-tocopherol to some samples of homogenized tissue (three samples of liver and one of heart) immediately before extraction and analysis. As is seen from Table 5, the addition of α-tocopherol did not materially affect the recoveries of the other lipid constituents. Statistical analysis of Table 5 shows that the standard deviation of the results amounts to about 3, 10 and 6 % of the mean for ubiquinone, ubichromenol and α-tocopherol respectively.

Statistical analysis of the results from the experiments on vitamin E

The data in Tables 2–4 illuminate an intricate series of relationships between the concentrations of the four lipid substances under varying conditions of age and nutrition, and the patterns exposed can obviously only be partially interpreted. Whilst the original design of the experiments was based on the requirements for an investigation into vitamin E, it soon became clear that the influence of this vitamin on the concentrations of ubiquinone and ubichromenol was of major interest and it was important to discover whether the changes were significant and to what degree. It was apparent, however, that the application of standard statistical designs to a study of such a complex nature as had been originally envisaged, which included tissue by tissue analysis of rats of both sexes at different ages and under varying nutritional regimens, would have involved a very large number of analyses, in addition to the several hundred already represented, so great in fact as to be virtually prohibitive. It seemed reasonable therefore to conduct a separate small-scale experiment, whose results would provide an estimate of variance that could be used as a first approximation to that probably occurring in the main investigation. Table 6 records the results of such an experiment, designed to test the variability, in respect of the quantities of the four lipid substances investigated, between groups of rats treated alike. The randomized groups were killed and the hearts and livers from each group were pooled and analysed.

The variance of each set of results for both sexes combined (organ weight, ubiquinone concentration etc.) was analysed in the usual way (Snedecor, 1946*a*) and a coefficient of variation (C) was calculated from the error mean square:

$$C = 100\sqrt{\frac{\text{error mean square}}{\text{mean}}}.$$

C was used to give an approximate 'least significant difference', in terms of percentages, between two groups of rats ($\pm Ct\sqrt{2}$), two pairs of groups ($\pm Ct$) and between two sets of three groups ($\pm Ct\sqrt{2}/\sqrt{3}$). Table 7 shows the data obtained in this way. Such estimates can be used only as a guide since variation in tissues other than heart and liver and in the tissues of older rats was not studied. Slightly less variability would be expected in the groups of 10–12 animals used in the main investigation.

Another approach to the problem of statistical significance in the main investigation is to treat some of the differences found as paired comparisons (Snedecor, 1946*b*); for example, the raised concentrations of ubiquinone found in hearts of all three

Table 2. *Distribution and interrelationships of vitamin E, vitamin A, ubiquinone and ubichromenol in 6-month-old male rats*

Analyses of depot fat are based on extracted lipid, not the weight of the crude tissue. Group A was on the vitamin E-deficient diet A40; group B was dosed with tocopherol before death; group C was on the supplemented diet A40E; group D was on the adequate diet SD1. n.d., Not detected; tr., trace.

Tissue	Group	Mean organ wt. (g.)	Ubiquinone (μg./g.)	Ubichromenol (μg./g.)	α-Tocopherol (μg./g.)	Vitamin A (i.u./g.)
Heart	A	1·20	41	12	2·3	0·9
	B	1·17	132	7	4·2	2·1
	C	1·22	168	31	34·1	11·0
	D	1·00	133	29	25·8	3·8
Liver	A	11·62	83	10	3·0	148
	B	11·20	62	13	9·6	160
	C	11·65	79	20	21·6	750
	D	8·06	107	42	14·1	177
Kidney	A	2·40	45	4	0·8	1·1
	B	2·41	72	6	6·0	3·9
	C	2·40	194	11	14·4	13·8
	D	1·60	78	11	8·9	3·6
Muscle (hind leg)	A	—	15	13	4·3	1·5
	B	—	37	3	8·3	3·0
	C	—	44	16	14·6	8·8
	D	—	40	18	12·6	—
Spleen	A	1·20	14	11	5·4	4·3
	B	1·09	21	13	23·4	5·7
	C	1·08	22	13	43·9	27·1
	D	1·55	17	16	14·2	—
Brain	A	1·89	14	6	3·7	9·5
	B	1·85	22	3	4·3	13·5
	C	1·96	19	4	6·3	6·6
	D	1·86	16	n.d.	6·5	—
Adrenal gland	A	0·056	89	92	68·4	71·4
	B	0·060	143	37	118	80·1
	C	0·060	132	67	218	104
	D	0·050	71	63	47·0	79·4
Lung	A	2·57	11	tr.	8·7	—
	B	2·43	16	3	14·2	—
	C	1·79	12	2	31·3	13·3
	D	1·78	6	5	13·5	4·2
Nerve	A	0·106	n.d.	n.d.	5·7	21·0
	B	0·114	22	n.d.	11·8	27·5
	C	0·103	21	8	25·7	7·2
	D	0·099	56	15	39·1	—
Testis	A	2·06	15	3	1·1	6·3
	B	1·77	25	3	4·8	11·2
	C	3·08	14	1	14·5	8·0
	D	2·78	7	n.d.	17·5	—
Fat	A	5·50	n.d.	n.d.	17·2	—
	B	5·89	n.d.	n.d.	16·5	—
	C	4·71	n.d.	n.d.	54·2	1·5
	D	2·70	tr.	25	108	—
Clot	A	—	2	5	2·1	0·2
	B	—	4	n.d.	4·5	—
	C	—	n.d.	3	2·9	0·2
	D	—	28	14	11·8	—
Serum	A	—	n.d.	n.d.	5·0	n.d.
	B	—	n.d.	n.d.	5·5	2·8
	C	—	n.d.	n.d.	1·8	—
	D	—	n.d.	n.d.	4·6	1·1

Table 3. *Distribution and interrelationships of vitamin E, vitamin A, ubiquinone and ubichromenol in 6-month-old female rats*

Analysis of depot fat are based on extracted lipid, not on the weight of crude tissue. Group E was on the vitamin E-deficient diet A 40; group F was dosed with tocopherol before death; group G was on the supplemented diet A 40 E; group H was on the adequate diet SD1. n.d., Not detected; tr., trace.

Tissue	Group	Mean organ wt. (g.)	Ubiquinone (μg./g.)	Ubichromenol (μg./g.)	α-Tocopherol (μg./g.)	Vitamin A (i.u./g.)
Heart	E	0·82	87	18	7·0	2·2
	F	0·77	159	15	12·6	3·9
	G	0·90	98	10	31·1	—
	H	0·78	158	20	34·0	5·3
Liver	E	7·42	90	53	4·3	323
	F	7·03	114	23	24·2	323
	G	7·29	88	39	36·2	500
	H	6·3	106	59	24·6	—
Kidney	E	1·46	97	6	4·8	4·1
	F	1·39	124	5	11·9	5·1
	G	1·47	49	11	18·3	18·4
	H	1·22	78	16	12·9	5·1
Muscle (hind leg)	E	—	31	5	5·4	1·7
	F	—	46	3	7·5	2·3
	G	—	46	22	27·1	—
	H	—	31	22	12·4	—
Spleen	E	0·80	18	11	10·1	1·3
	F	0·76	26	12	34·9	2·8
	G	0·70	50	18	33·8	—
	H	0·97	6	16	18·7	—
Brain	E	1·74	21	6	2·7	0·8
	F	1·72	28	4	4·2	1·1
	G	1·78	24	12	15·3	1·2
	H	1·75	21	n.d.	9·7	—
Adrenal gland	E	0·059	81	73	63·8	66·0
	F	0·061	130	44	176	86·0
	G	0·063	134	72	300	113
	H	0·050	139	81	285	77·5
Lung	E	1·56	6	3	4·9	13·5
	F	1·77	15	2	15·6	18·5
	G	1·61	13	11	33·6	12·1
	H	1·60	11	5	25·6	—
Nerve	E	0·097	10	tr.	28·5	10·1
	F	0·082	61	tr.	66·9	13·1
	G	0·077	95	24	104·3	30·2
	H	0·110	48	15	46·5	—
Uterus	E	0·71	17	9	8·2	3·1
	F	0·78	67	6	46·9	3·3
	G	0·67	18	7	68·6	4·3
	H	0·90	32	21	33·8	—
Fat	E	—	13	9	4·8	10·5
	F	—	n.d.	n.d.	6·9	2·9
	G	—	15	4	118	1·3
	H	—	tr.	22	161	—
Clot	E	—	n.d.	n.d.	n.d.	0·5
	F	—	tr.	tr.	4·2	1·0
	G	—	tr.	tr.	5·6	1·3
	H	—	27	15	15·4	—
Serum	E	—	n.d.	n.d.	0·6	—
	F	—	n.d.	n.d.	3·2	—
	G	—	n.d.	n.d.	1·6	—
	H	—	n.d.	n.d.	5·0	—

Table 4. *Distribution and interrelationships of vitamin E, vitamin A, ubiquinone and ubichromenol in 13-month-old female rats*

Analyses of depot fat are based on extracted lipid, not on the weight of crude tissue. Group J was on the vitamin E-deficient diet A 40; group K, also on A 40, was dosed with tocopherol before death; group L was on diet SD1. n.d., Not detected.

Tissue	Group	Mean organ wt. (g.)	Ubiquinone (μg./g.)	Ubichromenol (μg./g.)	α-Tocopherol (μg./g.)	Vitamin A (i.u./g.)
Heart	J	1·00	41	23	—	—
	K	1·00	106	14	5·7	0·6
	L	1·02	106	14	28·8	1·3
Liver	J	7·68	96	46	7·5	104
	K	7·92	114	71	20·7	102
	L	8·68	133	50	29·9	—
Kidney	J	1·60	86	14	1·2	1·7
	K	1·59	112	7	6·8	1·7
	L	1·58	90	8	25·9	—
Muscle (hind leg)	J	—	22	4	2·1	0·5
	K	—	30	2	6·3	0·6
	L	—	31	5	21·4	—
Spleen	J	0·94	21	8	3·0	2·0
	K	1·03	25	10	44·8	2·0
	L	1·09	31	46	30·6	—
Brain	J	1·74	5	5	1·0	n.d.
	K	1·77	14	6	3·6	n.d.
	L	1·90	29	5	11·1	—
Adrenal gland	J	0·067	122	178	13·9	104
	K	0·073	191	90	211	138
	L	0·069	128	84	264	102
Lung	J	1·92	7	4	8·1	4·2
	K	1·99	11	2	10·3	5·3
	L	1·93	6	4	20·4	5·2
Nerve	J	0·126	28	35	11·4	54·5
	K	0·098	65	18	27·6	9·0
	L	0·113	—	—	94·0	10·9
Uterus	J	1·43	6	4	n.d.	2·7
	K	1·18	22	3	24·6	3·6
	L	1·11	6	2	71·6	1·4
Fat	J	1·71	21	13	2·9	11·5
	K	1·29	78	8	28·1	—
	L	2·51	n.d.	n.d.	6·5	6·6
Clot	J	—	n.d.	3	3·1	n.d.
	K	—	4	1	5·8	0·2
	L	—	5	7	5·7	0·5
Serum	J	—	n.d.	n.d.	2·2	0·38
	K	—	n.d.	n.d.	7·3	—
	L	—	n.d.	n.d.	2·0	—

Table 5. *Precision of analyses and the effect of adding α-tocopherol to rat tissues*

Portions (5–10 g.) of each tissue were analysed. When tocopherol was added, it was added in an acetone solution directly to the acetone used for the initial homogenization and extraction.

Tissue	α-Tocopherol added (μg.)	Replicate analyses (μg./g.)		
		Ubiquinone	Ubichromenol	Tocopherol
Liver A	—	63·8	13·0	8·8
	—	61·7	11·0	9·6
Liver B	—	188	15·0	—
	250	181	15·8	—
Liver C	—	72·5	20·6	—
	250	73·7	23·6	—
Heart	—	204	15·2	16·3
	—	214	12·7	17·5

Table 6. *Measurement of variability between groups of 4-month-old vitamin E-deficient rats*

Expt. 1: Four groups of eight male rats, each group consisting of fully randomized litter-mates. Expt. 2: Four groups of eight male rats selected at random from a batch of 24 born within the same fortnight. Organs from each group were pooled and analysed. Figures given below represent the mean of each set of four results ± S.E.

Expt.	Organ	Organ wt. (g.)	Ubiquinone (μg./g.)	Ubichromenol (μg./g.)	α-Tocopherol (μg./g.)	Vitamin A (i.u./g.)
1	Heart	0·8 ± 0·031	211 ± 3·5	10·2 ± 0·25	4·2 ± 0·033	2·3 ± 0·05
1	Liver	13·0 ± 0·58	58 ± 2·0	20·7 ± 0·67	2·6 ± 0·07	—
2	Heart	0·74 ± 0·034	153 ± 2·1	14·1 ± 0·12	4·8 ± 0·11	3·5 ± 0·14
2	Liver	7·6 ± 0·71	105 ± 1·4	38·3 ± 0·30	3·5 ± 0·08	226 ± 2·2

Table 7. *Least significant percentage differences* calculated from the data in Table 6*

Least significant percentage differences (P 0·05) between	Organ wt.	Ubiquinone	Ubichromenol	α-Tocopherol	Vitamin A
Two lots of one group	51	11	10	13	11
Two lots of two groups	36	8	7	9	7
Two lots of three groups	30	6	6	7	6

* Calculated from the pooled data on heart and liver.

Table 8. *Changes in concentrations of ubiquinone and ubichromenol, where these occurred in the same direction in young male, young female and older female rats after oral dosage with vitamin E*

Changes in organs other than those listed did not occur in the same direction in all groups of rats.

Tissue	Mean increase* in ubiquinone (%)	P: From least significant difference (see Table 7)	P: From paired comparisons
Heart	57	<0·05	0·02
Kidney	26	<0·05	0·001
Muscle	40	<0·05	0·1
Spleen	26	<0·05	0·05
Brain	38	<0·05	0·01
Adrenal glands	37	<0·05	0·02
Lung	43	<0·05	0·1
Nerve	74	<0·05	0·05

Tissue	Mean increase* in ubichromenol (%)		
Heart	−33	<0·05	0·1
Muscle	−64	<0·05	<0·25
Spleen	14	<0·05	0·05
Adrenal glands	−50	<0·05	0·1

* Related to the higher figure in each case.

groups of rats when dosed with α-tocopherol may be treated thus, and the increment over the concentrations in the controls proves to be significant at P 0·02. A number of such changes in ubiquinone and ubichromenol (these being the changes of prime interest), occurring consistently in the tissues of the three groups of rats after oral dosage with vitamin E, are set out in Table 8 and their significance is assessed by both methods. In several cases, the paired-comparison method gave a lower degree of significance than the other method, possibly due to the larger value of t (4·3) for the former, compared with t 2·26 for the latter. A comparison between the effects of diets A 40 and A 40 E was made only with the younger rats, and the changes in the concentrations of ubiquinone and ubichromenol are recorded in Table 9. Differences of 8 and 7 % respectively would probably be significant (see Table 7). The changes in these two substances have been dealt with in more detail because of the biochemical implications. The significance of variation in concentrations of vitamins E and A may be judged by reference to the least significant differences set out in Table 7.

Distribution of vitamin E in the rat

Although several figures already exist in the literature for the vitamin E content of rat tissues (Quaife, Swanson, Dju & Harris, 1949; Swick & Baumann, 1952), certain tissues recorded in the present Tables do not appear to have been studied before. These are erythrocytes, nerve and (surprisingly) uterus. This also seems to be the first study of tissues from the vitamin E-deficient rat, where levels are generally below the range amenable to study by earlier workers.

Younger rats on stock diet SD 1. The particular interest of this group of analyses is that they may be taken as representing those found in the normal healthy adult rat. It is at once apparent that there is a considerable sex difference with respect to vitamin E. There was a greater concentration of tocopherol in every tissue taken from females than in the corresponding tissue from the males. Even when the results are recalculated on a 'whole-organ' basis, so as to compensate for the greater weights of the organs in the males (see Tables 2–4). it is still clear that the female rat contains much

Table 9. *Magnitude and significance of changes in concentration of ubiquinone and ubichromenol in tissues of 6-month-old male and female rats on supplementing the deficient diet with vitamin E*

A change of 7–8% is probably significant (see Table 7).

Tissue	Mean increases (%)* (A 40 E compared with A 40)	
	Ubiquinone	Ubichromenol
Heart	52	†
Liver	−3	†
Kidney	†	55
Muscle	49	53
Spleen	55	29
Brain	19	†
Adrenal glands	36	−16
Lung	32	77
Nerve	91	100

* Related to the higher figure in each case.
† Change different in different sexes.

more vitamin E than the male. The tissues themselves show considerable variation in tocopherol content. High concentrations occurred particularly in adrenal gland, nerve, heart and uterus. As already shown by Quaife *et al.* (1949), the rat adrenal gland is extraordinarily rich in α-tocopherol. In the present series of experiments the female adrenal gland (whether from stock-colony, vitamin E-deficient or vitamin E-supplemented animals) was found to contain several times more vitamin E than any other tissue.

Younger rats on deficiency diet A 40. The tissues from 6-month-old rats on the vitamin E-deficient diet showed certain differences from those of stock animals. The organs depleted at different rates, resulting in a partial redistribution of vitamin E in the body. The vitamin E-deficient male adrenal glands, for instance, contained more tocopherol than those from animals on diet SD 1. It seemed that female rats depleted more readily than males; this is shown by the fact that five tissues (including the depot fat) in the deficient males contained somewhat more vitamin E than those in the females. No tocopherol could be detected in blood clot from the deficient females, although some was still present in the serum. Major decreases in tocopherol occurred in male testis and kidney during depletion.

Younger rats on supplemented diet A 40 E. The tissue analyses from the 6-month-old animals on this diet also present some interesting features. The diet provided about ten times as much vitamin E as the stock diet SD 1, but uptake by the tissues was variable and, to some degree, selective. In the female, large increases of vitamin E were observed in uterus (compared, for instance, with skeletal muscle) and in nerve and adrenal gland. In general, most tissues from females on A 40 E contained more tocopherol than those from females on diet SD 1. However, the difference in the composition of the two diets obviously had a profound effect on the uptake of vitamin E: in spite of the high concentrations of tocopherol in A 40 E and in many organs of the rat fed on this diet, the tissues concerned with transport and storage of the vitamin, i.e. serum, fat and, possibly, erythrocytes, contained concentrations of tocopherol that were quite low compared with those in tissues from stock rats. In the male rats on A 40 E, only the adrenal gland showed an unusually high tocopherol level, although spleen and lung contained amounts in excess of normal. Males did not show the big increase in the sciatic nerve, and, in general, the male tissues (including fat and testis) had a lower concentration of vitamin E than did rats on the stock diet. Male serum, however, contained more tocopherol than female serum.

Administration of vitamin E to deficient animals. Dosage of male and female rats with α-tocopherol 48 hr. before death increased the concentrations in nearly all tissues (fat, as might be expected, being hardly affected). The major increases in the male were in spleen and adrenal: heart, brain and liver showed only small changes. Female tissues, in general, showed bigger increases than male tissues. Exceptionally big increases were again found in female adrenal gland, nerve and uterus.

Older rats. The tissues of 13-month-old female rats showed considerable differences compared with those from the younger animals. Comparing, first, animals on the stock diet, although several tissues in the older rats contained less tocopherol than those from the younger ones, the only really big change was in fat, in which a dramatic drop in the vitamin E reserves was apparent. Muscle, spleen and, in particular, nerve and uterus showed big increases on aging. When the two sets of deficient animals are compared (on diet A 40), it will be seen that, in most cases, the tissues of the older rats contained considerably less vitamin E than the younger ones. Uterus at 13 months contained no detectable tocopherol. Administration of 10 mg. of α-tocopheryl acetate to the older animals produced effects similar to those in the younger ones, the biggest increases being in adrenal gland and uterus.

Relationships between vitamins A and E in experimental vitamin E deficiency

It is well known that vitamin A levels in the rat are affected by the amounts of vitamin E in the diet. Since the studies of Morton and co-workers (see, for example, Morton & Phillips, 1959*a*) have indicated that concentrations of ubiquinone and ubichromenol in rat liver are influenced by vitamin A,

throughout most of the present series of experiments vitamin A was measured in the tissues whenever this was possible, and the results are recorded in Tables 2–4. As was to be expected from the findings of earlier workers, for example Moore (1939), the vitamin A of most tissues fell in concentration during vitamin E deficiency (although in adrenal gland and lung little change was observed). Administration of tocopherol before death caused appreciable increases of vitamin A in nearly all the tissues with the exception of fat and liver, the effects being similar in both sexes.

Ubiquinone and ubichromenol in the stock-colony rat

Although a few isolated reports have already appeared dealing with concentrations of ubiquinone in the rat, these have so far been restricted to a few tissues. Moore & Sharman (1959) and Moore (1960) reported figures for heart and liver, but the values are much higher than any found in our experiments: Moore did not use either chromatographic purification or borohydride assay, however. Morton & Phillips (1959*a*, *b*) examined liver, kidney, heart and testes and the concentrations reported by them for stock animals are of the same order as found in our series.

To compare the quantitative nature of our results with those of Morton & Phillips, we have compared our extraction and saponification procedure with that of these workers, from which it differs in several respects. A quantity of pooled rat livers was minced and divided into several 10 g. portions. These were saponified and extracted by several methods as described in Table 10, which records the concentrations of ubiquinone, ubichromenol and tocopherol determined by each method. The method of Mervyn & Morton (1959), in which the total tissue is saponified, gave good agreement with our standard method, in which the lipid extract only is treated for 5 min. under reflux with ethanolic potassium hydroxide. When the ethanol used in the method of Mervyn & Morton was replaced by Grignard-purified methanol, the recovery of ubiquinone was excellent, but the ubichromenol figure was lower. Saponification of the tissue with aqueous alkali gave a poor recovery of all three substances. Methanolic potassium hydroxide cannot be used successfully on the extracted lipid. Major differences in the recovery of ubiquinone and ubichromenol (but not of α-tocopherol) were found when ordinary analytical reagent-grade methanol was substituted for the specially purified methanol and some caution is obviously necessary when this reagent is used. Comparison of the ubiquinone fractions obtained from these experiments indicated that the compound previously tentatively identified by us as a dihydroubiquinone (Diplock *et al.* 1960*a*) may have been a small amount of the ethoxy compound produced during the saponification process (Linn, Trenner, Shunk & Folkers, 1959).

As shown in Tables 2–4, the highest amounts of ubiquinone in animals on diet SD1 were found in heart at 6 months of age; in the older females the amount in heart had decreased considerably, more than was compensated for by the increase in weight of the organ. High concentrations of ubiquinone were observed in liver, and also in adrenal, nerve and kidney. Uterus contained considerably more ubiquinone than testis at 6 months, and at 13 months, the uterus was heavily depleted of ubi-

Table 10. *Effect of various methods of saponification of vitamin E-deficient rat liver on the recovery of ubiquinone, ubichromenol and α-tocopherol*

Portions (10 g.) of a pooled batch of liver were used, and in Expts. 1–5 they were saponified directly according to Mervyn & Morton (1959); in Expts. 6–9 they were extracted and then saponified with the reagents described in the Table. Each non-saponifiable extract was separately analysed, after removal of sterols and treatment on Decalso, by two-dimensional paper chromatography.

Expt. no.	Conditions	Saponification time (min.)	Ubiquinone (μg./g.)	Ubichromenol (μg./g.)	α-Tocopherol (μg./g.)
1	Ethanol (4 ml.)–KOH (1 ml.)	3	33·9	26·9	2·2
2	Ethanol (4 ml.)–KOH (2 ml.)	5	35·2	26·9	2·4
3	Purified methanol* (4 ml.)–KOH (2 ml.)	5	23·1	26·9	2·1
4	Methanol† (4 ml.)–KOH (2 ml.)	5	7·7	3·3	3·6‡
5	Water (4 ml.)–KOH (2 ml.)	10	8·2	8·7	1·2
6	Total tissue with ethanol	60	36·2	25·7	2·2
7	Total tissue with methanol†	60	34·2	10·2	3·7‡
8	Total tissue with purified methanol*	60	26·0	27·3	1·9
9	Total tissue with water	60	11·7	10·0	2·4

* Methanol (A.R.) purified by treatment with methyl magnesium iodide, followed by fractional distillation.
† Methanol (A.R.) from drum.
‡ Colours increased continually during Emmerie–Engel determination. This was often noticed after other saponifications with methanolic KOH.

quinone although, it should be noted, the tocopherol content had increased. As with vitamin E, the female tissues generally contained more ubiquinone than did the male tissues, the main exception being found in spleen. Ubiquinone was found in blood clot in considerable quantities, but none was found in serum and only traces could be detected in body fat. More recent work, however, has indicated that washed erythrocytes do not contain appreciable amounts of ubiquinone, and, in all probability, the amounts recorded in blood clot were derived from leucocytes.

Ubichromenol was found in all tissues except serum. Concentrations were usually lower than corresponding concentrations of ubiquinone in the same tissue and were highest in adrenal gland and liver in both sexes. Fat from both sexes contained appreciable quantities of ubichromenol, although very little ubiquinone could be detected: this was the only tissue in which this occurred.

Effect of dietary vitamin E on ubiquinone and ubichromenol

With a few exceptions, concentrations of ubiquinone were found to be significantly lower in the vitamin E-deficient animals on diet A 40, compared with either stock-colony animals or those on the supplemented diet A 40 E (see Tables 7 and 8). In the younger rats, vitamin E deficiency produced substantial falls in the ubiquinone level in sciatic nerve, spleen and adrenal gland. Some tissues showed little change, whereas female kidney (6 months) and male adrenal gland and testis showed small increases. With testis, changes in organ weight under the influence of vitamin E tend to obscure the interpretation of the ubiquinone concentrations. Ubichromenol concentrations were also decreased in most tissues of the vitamin E-deficient 6-month-old rat, the only exceptions being brain in both sexes and adrenal gland and testis in the male. In the old females, higher concentrations of ubichromenol were found in some of the vitamin E-deficient tissues when these were compared with stock-colony animals.

Effect of vitamin E administration before death

Administration of 10 mg. of DL-α-tocopheryl acetate 72–48 hr. before death increased the concentrations of vitamin E in all tissues of the three groups investigated. Particularly big increases occurred in uterus, adrenal glands, spleen and, in the younger females, sciatic nerve. Vitamin E administration produced significantly increased concentrations of ubiquinone in most tissues studied (see Table 8); the chief exceptions found were in fat from 6-month-old females and in liver from the males. The largest increases were in uterus, heart and nerve: the concentration changes in liver were relatively small.

The increase in ubiquinone was in most tissues accompanied by a decrease in ubichromenol (see Table 8) but this did not occur consistently in all three experiments. A few exceptions were observed: spleen showed a significant increase in ubichromenol, and male liver, almost the only tissue in which ubiquinone decreased, also showed an increase in ubichromenol.

Effect of dietary selenium on ubiquinone and ubichromenol

The role of dietary selenium in its relation to vitamin E-deficiency conditions is not understood;

Table 11. *Effect of dietary selenite on ubiquinone and ubichromenol in the tissues of the rat*

Groups M and O were fed on the necrogenic diet. Groups N and P were fed on the necrogenic diet + selenite. Group Q was fed on the necrogenic diet + α-tocopherol. Group R was fed on the necrogenic diet + α-tocopherol and selenite. Group S was fed on diet A 40. Group T was fed on diet A 40 + selenite.

Expt. no.	Tissue	Group	Ubiquinone (μg./g.)	Ubichromenol (μg./g.)	α-Tocopherol (μg./g.)
1	Heart	M	99·7	*	2·6
		N	157	*	1·9
	Kidney	M	45·9	*	0·3
		N	62·9	*	0·5
2	Heart	O	84·6	9·5	6·9
		P	136	22·2	5·5
		Q	92·9	7·1	18·7
		R	128	9·2	20·2
	Liver	O	35·6	8·9	2·6
		P	57·1	18·7	3·4
		R	71·6	16·4	16·4
3	Heart	S	207	10·2	7·5
		T	205	13·6	7·3
	Liver	S	101	17·7	2·3
		T	233	29·2	4·0

* Present but not measured.

but, since the identification of Factor 3, which prevents liver necrosis in rats, with natural selenium compounds (Schwarz & Foltz, 1957), a close and growing correspondence between selenium and α-tocopherol in preventing nutritional deficiencies has been recognized. It was therefore of interest to study the influence of selenium on concentrations of ubiquinone and ubichromenol.

Table 11 records the results of the three experiments carried out, details of which have been given in an earlier section. It was found that the addition of selenium to the necrogenic diet significantly increased the amount of ubiquinone in the two tissues examined (heart and kidney) in the first experiment. In the second experiment, selenium added to the necrogenic diet significantly increased the amount of ubiquinone in heart and liver. In the third experiment, selenium added to a vitamin E-deficient diet (non-necrogenic) increased ubiquinone in liver, but not in heart.

DISCUSSION

The vitamin E-deficient diet (A 40) used in these experiments was found to contain 2·4 μg. of α-tocopherol/g. As far as we are aware, this is the first direct determination to be made of a tocopherol-deficient rat diet, and the figure is surprisingly high, if it is compared with that obtained on the nutritionally adequate diet, SD 1 (9·5 μg./g.). Most of the tocopherol in the deficient diet evidently comes from the lard. Lest it be thought that the type of lard used contained unduly large amounts of tocopherol, we have in fact examined several 'pure' lards and found them to be similar in tocopherol content. The extracted casein also contained tocopherol. The discovery of these hitherto unsuspected amounts of tocopherol in constituents believed to be practically vitamin E-free is a direct consequence of the substitution of Decalso for activated floridin earth in the purification stage of vitamin E analysis. Although floridin-earth purification is suitable for the analysis of vegetable oils (Report of the Vitamin E Panel, S.A.C., 1959) and normal animal tissues (Edwin *et al.* 1960), there seems no doubt that it must now be regarded as unsuitable for the determination of tocopherol in exceptionally low concentrations, such as occur in vitamin E-deficient animal tissues and purified diets and fats. As a further example of this, we have analysed the 'stripped' lard, especially prepared by Distillation Products Industries (U.S.A.) for use in vitamin E-free diets. Bieri, Briggs, Pollard & Fox (1960) reported less than 0·5 μg. of tocopherol/g. in this material by the use of a method essentially similar to ours, but using activated Florex for purification. When we determined the α-tocopherol on the unsaponifiable fraction from 20 g. of this lard, with Decalso followed by paper chromatography, we found 3·3 μg./g. (as such, it should be pointed out, it was the most tocopherol-free lard we encountered and fell well within the manufacturers' declaration).

In spite of the amount of tocopherol in diet A 40, this diet produced all the usual symptoms of vitamin E deficiency in our rats. Presumably it contained less vitamin E than the standard bioassay diet recommended by Mason & Harris (1942), which contained up to 10% of olive oil (a rich source of tocopherol by these standards).

These findings confirm what is already recognized, that the requirements for vitamin E are dependent on both the quantitative and qualitative aspects of dietary composition, particularly, but probably not only, with respect to fat. This is generally reinforced by the results of the present series of experiments, which show that deposition of tocopherol in the adipose tissue of the rat is profoundly affected by the type of diet, more so than by the amount of tocopherol ingested. It follows from these experiments that vitamin E status in the rat cannot be simply defined. It must certainly be based on actual tissue analyses, and it would seem, further, that those most usually carried out (serum or fat analyses) might not, at least in the rat, be the most helpful. A better guide to vitamin E status would appear to be the analysis of heart muscle.

The rat is able to take up large amounts of α-tocopherol from its diet (or from an administered dose) and incorporate them into its tissues. This ability is not so unremarkable as it might seem: in succeeding papers it will be shown that it is a characteristic not found in all species. The data presented in Tables 2–4 show that the tissues of the rat vary considerably in tocopherol uptake, and this is so in both sexes. Adrenal gland and nerve, particularly the former, accumulate exceptionally large concentrations of tocopherol, and it may be noted that these concentrations tend to persist into extreme vitamin E deficiency (which, in the rat, is marked by virtual exhaustion of the tocopherol stored in the fat). If these high concentrations of tocopherol in the adrenal gland are considered in conjunction with an earlier finding of Quaife *et al.* (1949), who showed that the concentration of tocopherol in rat pituitary was three times as great as that in the adrenal gland, it is tempting to speculate that there is a specific physiological need for the vitamin connected with high metabolic activity of these two organs. If vitamin E is simply acting as an intracellular antioxidant, highest concentrations might well be expected in those tissues where high rates of metabolic turnover would conceivably give rise to other labile intermediates whose removal

from the cell was essential. However, such a simple approach is certainly not encouraged by the finding that both liver and kidney take up only moderate amounts of tocopherol and deplete very readily; organs of low metabolic activity such as uterus and nerve build up high concentrations of tocopherol.

In view of our present findings, it is essential to examine the 'antioxidant' theory of vitamin E more extensively. Several workers, especially Tappel & Zalkin (1959*a*, *b*) and Zalkin & Tappel (1960), have suggested that the biological role of vitamin E is limited to that of an intracellular antioxidant. Two functions are usually considered: (*a*) the protection of other essential but sensitive substances, such as vitamin A and unsaturated fats, and (*b*) the removal of free-radical intermediates produced by the oxidation of unsaturated lipids, which might react with and damage other cellular components, such as members of the electron-transport chain. With regard to the first type of function, although it is clear from our results that vitamin E has a sparing effect on the vitamin A in the tissues, there does not appear to be any regular correlation in the concentrations of these two substances. It is true that adrenal gland contains much vitamin A, but liver contains much more, and there is no evidence of high vitamin E concentrations in those organs that contain exceptional amounts of vitamin A, such as lung or kidney. There are more serious difficulties about accepting the conclusions of Tappel & Zalkin for the second function. First, the formation of lipid 'peroxides' (as measured by the thiobarbituric acid reagent) in various tissues cannot, it seems to us, be related to the vitamin E content of these tissues, nor can the somewhat limited effect of tocopherol in preventing peroxidation be simply related to the amount or type of unsaturated lipid in the tissues. Secondly, the specific effects of vitamin E deficiency in animals, which vary according to the species under study, cannot be related to the action of tocopherol in preventing tissue peroxidation. Thus in the rabbit enhanced peroxidation has been demonstrated by Zalkin & Tappel in vitamin E-deficient liver and kidney, but not in muscle, which in this animal is the main tissue affected by vitamin E deficiency (see, however, Carpenter, Kitabchi, McCay & Caputto, 1959). Similarly, Carpenter *et al.* have shown that although rat liver is subject to peroxidation and that this can be prevented by tocopherol, rat testis, the organ in the male chiefly affected by vitamin E deficiency, is readily peroxidized and this cannot be prevented by vitamin E. Pritchard & Singh (1960) related the extent of peroxidation of several rat tissues to the polyunsaturated fatty acid content of the tissues. They showed (*a*) that adrenal gland was extraordinarily sensitive to peroxidation and (*b*) that neither the peroxidation nor its prevention by vitamin E could be simply related to the polyunsaturated fatty acid composition of the tissue. It would appear therefore that the organ most susceptible to lipid peroxidation in the rat is the one that, according to our findings, contains more vitamin E than any other organ in the body, whether from deficient or supplemented animals. Pritchard & Singh also showed that vitamin E-deficient liver was the tissue least susceptible to peroxidation, and, again, if our findings are relevant, this is a tissue that contains one of the lowest tocopherol concentrations in the body. Pritchard & Singh found that brain peroxidation was very high and could not be prevented by administration of vitamin E. Our results show that, although brain contains relatively small amounts of vitamin E, tocopherol passes the blood–brain barrier [compare Bieri (1960), who has suggested that in chicks this might not be so, and Abderhalden & Elsaesser (1946), who found no tocopherol in human cerebrospinal fluid]. Unpublished work in this laboratory has confirmed that high levels of peroxidation are normal in the brains of adequately fed rats and chickens, indicating that the 'peroxidation' measured by the thiobarbituric acid reagent, at any rate, is not necessarily either an abnormal or deleterious phenomenon in tissues. In this connexion, although Zalkin & Tappel consider that the cellular damage produced by vitamin E deficiency takes place in the mitochondria, where most of the unsaturated lipid is, Pritchard & Singh found that most of the peroxidation in rat brain and heart took place in the microsomes and supernatant fraction (see also Carpenter *et al.* 1959). It would be interesting to compare the peroxidation of nerve tissue with brain in the rat, in view of the completely different amounts of vitamin E in these two tissues.

A further point of interest that emerges from the data on vitamin E distribution is the significant response of uterus both to depletion of and to administration of vitamin E. In view of the nature of this organ, whose tissue has relatively little metabolic or synthetic activity, it would appear reasonable to associate this property with its potential function, that is, with the requirements of foetal development. It is known that vitamin E can be transferred across the rat placenta only with difficulty (Mason & Bryan, 1938). It is possible therefore that the uterine reserves of tocopherol in the rat may be needed for transfer to the foetus in the early stages of development. The significance of this concept is that such a deficiency might be not only relative but local, in the sense that it could arise as a unique manifestation of the deficiency state as a result of an abnormally high tocopherol

requirement of the young foetal tissue, and whilst other tissues in the body still contained sufficient tocopherol for their needs. This possibility that foetal rather than solely uterine requirements for tocopherol are involved in the phenomenon of gestation-resorption in the rat receives some support from observations made by various workers in the classical studies of the condition. Among these may be mentioned the fact that tocopherol can prevent resorption if given during the first week of pregnancy (Mason, 1942), the absence of an influence of uterine pigmentation or other uterine changes on subsequent pregnancy, provided that vitamin E is given (Evans & Emerson, 1943), and the observed anatomical and histological phenomena associated with foetal death, which include diminished haemopoiesis and asphyxia due to failure of the maternal–foetal vascular relationship (Evans & Burr, 1927; Urner, 1931; compare the histological manifestations of encephalomalacia in the chick, which also involves vascular degeneration, described by Wolf & Pappenheimer, 1931). It may be speculated whether the specific manifestations of vitamin E deficiency in other species may not similarly reflect local tissue deficiencies of tocopherol.

Concentrations of ubiquinone and ubichromenol also show large variations from tissue to tissue, and, in general, are highest in those tissues that are richest in tocopherol.

The statistical treatment of the data leads to the conclusion that deficiency of vitamin E has a marked influence on the concentrations of ubiquinone and ubichromenol in the rat. The rapid effect of oral administration of vitamin E in restoring ubiquinone and ubichromenol concentrations in the tissues suggests that a biosynthetic relationship exists between the three substances, although this may be of an indirect nature. There appears to be no obvious connexion between these findings and any auxiliary function that tocopherol may have as a physiological antioxidant. If tocopherol were 'sparing' ubiquinone in the same manner as, at a lower level of concentration, it spares vitamin A, it is difficult to see why it does not spare ubichromenol, which we have found to be even more sensitive to oxidation than the quinone.

If 'antioxidant' theories are insufficient to account for the action of vitamin E, it is necessary to look elsewhere for an explanation of its effect on ubiquinone. Gray (1959) has shown that, in rats, dietary supplementation with vitamin E increases the production of cholesterol by liver homogenates *in vitro*. A similar effect might well be involved in the synthesis of the ubiquinone side chain, which is derived, like cholesterol, from mevalonic acid [the effect of vitamin E in counteracting hypercholesterolaemia in the rabbit (Smith, Shin & Freier, 1960; Draper, 1959) and decreasing concentrations of cholesterol produced by exogenous cholesterol in the rat (Aftergood, Deuel & Alfin-Slater, 1957) is due to another mechanism, the control of unsaturated fatty acid uptake (see, in this connexion, Pritchard & Singh, 1960)]. A number of workers have tentatively implicated vitamin E with oxidative phosphorylation, and Chernick, Moe, Rodnan & Schwarz (1955) suggested that its effect may be on adenosine triphosphate (ATP) synthesis. The recent demonstration by Gray, Chisholm & Lee Peng (1960) that dietary supplementation with α-tocopherol significantly increases liver synthesis of ATP and coenzyme A in the rat must be carefully considered. Gray *et al.* (1960) have proposed a hypothetical scheme of synthesis for ATP, which involves tocopherol in phosphorylation reactions. It seems germane to ask, does the increased synthesis of ATP lead to the increased synthesis of substances such as cholesterol, coenzyme A and ubiquinone; or, does tocopherol have a more specific influence on ubiquinone synthesis, which, by increasing oxidative-phosphorylation processes, would in turn lead to an increase in ATP? It may be significant that, in the present experiments, the influence on ubiquinone was observed with only one-hundredth of the dietary α-tocopherol used by Gray *et al.* (1960) (but compare the difference in dietary composition). Whatever the mechanism, though, such an effect of tocopherol on ubiquinone must have other consequences. The latter substance is an essential component of the electron-transport chain, connecting the flow of electrons from substrate to the cytochromes (Hatefi, Lester, Crane & Widmer, 1959). Tocopherol therefore, by an effect on ubiquinone concentration, could affect electron transport. Such a role would make relevant and understandable many of the earlier findings that have linked vitamin E with respiration processes (for example, see Hove, Hickman & Harris, 1945; and Taylor, 1953). Subsidiary effects, for example on the synthesis of ascorbic acid (Caputto, McCay & Carpenter, 1958), might also be related.

Study of the data does not disclose any obvious connexion between concentrations of ubiquinone and vitamin A in the various tissues. Morton and his co-workers have discussed in several papers the relation between concentration of ubiquinone and vitamin A deficiency in the rat (for example, Lowe, Morton & Harrison, 1953; Heaton, Lowe & Morton, 1957), and it seems that the matter must now be more fully elucidated in terms of the complex relationship between vitamins A and E. Morton & Phillips (1959*b*) found that vitamin E had no effect on amounts of ubiquinone in rat liver, heart, kidney or testis. While we are unable to account for the difference between their results and ours,

there were not only great differences in analytical technique, but the vitamin A status of the animals was quite different in the two sets of experiments.

The effect of dietary selenium in increasing concentrations of both ubiquinone and ubichromenol is similar to the effect of dietary tocopherol. There are two additional points of interest. Whereas the effect of tocopherol is usually small in liver, that of selenium appears to be more pronounced. This may have some bearing on the role of selenium in preventing nutritional liver necrosis in rats (Schwarz & Foltz, 1957), whereas it is ineffective in preventing gestation-resorption (Harris, Ludwig & Schwarz, 1958). Secondly, dietary selenium was obviously able to enhance ubiquinone concentrations (in liver, at least), even though the basal diet must be assumed to have contained adequate amounts of Factor 3 (organic selenium compounds), because of its casein content. The observations already made, however, confirm the duality of the biochemical roles of selenium and α-tocopherol and render it unlikely that the biological role of the latter can be understood in terms of its function as a physiological antioxidant.

SUMMARY

1. The distribution of vitamin E, vitamin A, ubiquinone and ubichromenol has been studied in 14 tissues of the rat. Tissues richest in vitamin E are usually richest in ubiquinone. High concentrations of vitamin E and ubiquinone are present in adrenal gland, heart, uterus and nerve.

2. The vitamin E content of the tissues is affected by the nature of the diet.

3. Tissues lost their vitamin E at different rates during depletion. Tissues such as adrenal gland and nerve retain high concentrations of tocopherol, whereas uterus and liver readily lose their tocopherol. The susceptibility of uterus to tocopherol depletion and administration is related to the gestation-resorption syndrome in the rat.

4. The distribution of the four substances has been compared in 6-month-old and 13-month-old female rats and in males and females at 6 months.

5. Female rats contain higher amounts of the four substances than male rats of the same age.

6. Male and female vitamin E-deficient rats have lower concentrations of ubiquinone in their tissues than either the corresponding animals whose diet has been supplemented with vitamin E or animals on the stock diet. Oral administration of vitamin E to such deficient animals increases the ubiquinone concentration in their tissues. This increase in ubiquinone is often accompanied by a decrease in ubichromenol concentration, which tends to be elevated in the vitamin E-deficient rat.

7. Dietary selenium increases the concentration of ubiquinone in the same way as does α-tocopherol. It seems that there is a biosynthetic relationship between tocopherol, selenium, ubiquinone and ubichromenol.

8. The specific syndrome of vitamin E deficiency in animals may be related to a local tissue deficiency of this vitamin.

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Studies on Vitamin E

7. THE EFFECT OF THIAMINE, RIBOFLAVIN AND PANTOTHENIC ACID ON UBIQUINONE AND UBICHROMENOL IN THE RAT

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In the preceding paper, Edwin, Diplock, Bunyan & Green (1961) have shown that concentrations of ubiquinone and ubichromenol in the tissues of the rat are dependent on vitamin E status. It was of interest to determine whether this effect was specific to vitamin E, or whether concentrations of ubiquinone in the rat were generally influenced by nutritional status, in particular by other vitamins. In the present work, the effect of thiamine, riboflavin and pantothenic acid on ubiquinone was studied.

EXPERIMENTAL

Animals, diets and analyses

Male and female rats of the Norwegian hooded strain were used. They were removed from the stock colony at 4–5 weeks of age and placed on the test diets. They were killed with ether.

Basal diet. This had the following composition: casein (Genatosan Ltd. 'low-vitamin content'; 25%), sucrose (50%), glucose (10%), arachis oil (10%), a vitamin mixture (0·4%) (to supply per kg. of diet: nicotinic acid, 90 mg.; pyridoxine, 9 mg.; folic acid, 2 mg.; calcium pantothenate 90 mg.; vitamin B_{12}, 0·03 mg.; inositol, 90 mg.; *p*-aminobenzoic acid, 90 mg.; choline hydrogen tartrate, 1·8 g.; menaphthone, 20 mg.) and Cox's salt mixture (5%) to provide (g./kg. of diet): NaCl, 2·7; $CaH_4(PO_4)_2$, 21·7; ferric citrate, 1·9; NaH_2PO_4, 5·5; $MgSO_4,7H_2O$, 4·3; KCl, 13·6; KI, 0·055; NaF, 0·0021; $MnSO_4,4H_2O$, 0·011. Vitamin A, vitamin D_3 and α-tocopheryl acetate were added to supply respectively 4000 i.u., 400 i.u. and 120 mg./kg.

Thiamine-deficient diet. This was the basal diet to which had been added riboflavin, 19 mg./kg., and calcium pantothenate, 90 mg./kg.

Riboflavin-deficient diet. This was the basal diet to which had been added thiamine, 9 mg./kg., and calcium pantothenate, 90 mg./kg.

Pantothenic acid-deficient diet. This was the basal diet to which had been added thiamine, 9 mg./kg., and riboflavin, 18 mg./kg.

Experimental plan and analyses. The experiments on thiamine and riboflavin deficiency were carried out simultaneously with one group of controls. Rats were divided into groups of eight (equal numbers of males and females in each) at 28 days of age. Group A received the basal diet supplemented with thiamine and riboflavin. Groups B and C received the thiamine-deficient diet, groups D and E the riboflavin-deficient diet. The groups were maintained on their diets for 38 days, by which time the deficient groups were showing weight losses. At 72 hr. before the end of the experiment, and again at 48 hr., group C rats were given oral doses of 0·5 mg. of thiamine and group E rats were given oral doses of 1·25 mg. of riboflavin. A similar experiment was devised for pantothenic acid deficiency. Three groups of rats were used (10 in each group, equal numbers of each sex). Group F received the deficient diet supplemented with calcium pantothenate, groups G and H the deficient diet. At the end of the experiment (28 days),

group H rats were given two oral doses of 3 mg. of calcium pantothenate. The amount of these oral doses was decided, in each case, from a computation based on the known requirements of the rat for these three vitamins. Roughly, as much was given in the total oral dose as would have been required by the rat during the test period.

In a final experiment, the simultaneous effect of vitamin E and pantothenic acid deficiency was studied. The rats (48 males) were randomized into eight groups of six and fed on the following diets: groups J, K, L and M were given the doubly deficient diet, group N was given the diet supplemented with 100 mg. of calcium pantothenate/kg., group O the diet supplemented with 100 mg. of DL-α-tocopheryl acetate/kg., group P the diet supplemented with both calcium pantothenate and the tocopheryl acetate, and group Q the diet supplemented with 500 mg. of DL-α-tocopheryl acetate/kg. After a month on test, the rats in group K were each dosed orally with 12 mg. of calcium pantothenate, those in group L with 10 mg. of α-tocopheryl acetate and group M with the same amounts of both substances. The doses were given in two portions, 72 and 48 hr. before death. All the rats were then killed. The tissues within each group were individually weighed and then pooled and stored at −20° until ready for analysis (usually 2 or 3 days). They were analysed for tocopherol, vitamin A, ubiquinone and ubichromenol by the method of Diplock, Green, Edwin & Bunyan (1960).

RESULTS AND DISCUSSION

The results of the experiments on thiamine and riboflavin deficiency are given in Tables 1–3. The concentrations of ubiquinone, ubichromenol and α-tocopherol remain remarkably constant in rat heart and do not appear to be affected either by dietary supplementation or by oral dosage with either of the two vitamins 48 hr. before death. The total amounts of the three lipid constituents in the whole heart were, however, considerably less in the deficient animals, compared with supplemented controls. In liver there is apparently a slight decrease in the concentration of both ubiquinone and ubichromenol during riboflavin deficiency and an overall loss from the organ (773 μg. against 1089 μg. for the supplemented animals). The fluctuation in liver weight, however, makes it difficult to decide whether these differences are truly significant. However, there was a big decrease in the α-tocopherol concentration in the deficient livers compared with controls: this is in clear contrast with the situation in heart. Dosage with riboflavin did not affect the concentrations of ubiquinone, ubichromenol or α-tocopherol. The most striking difference was found in thiamine-deficient liver, where the concentrations of ubiquinone and ubichromenol, but not of tocopherol, were nearly doubled. Dosage with thiamine promptly decreased the ubiquinone and ubichromenol concentrations to those of the controls and halved the tocopherol concentration. Very large weight changes in the liver took place, however, during thiamine deficiency, as the figures show, and the total amounts of each substance in the

Table 1. *Effect of thiamine and riboflavin on concentrations of ubiquinone and ubichromenol in rat heart*

Group and treatment	Mean final rat wt. (g.)	Mean organ wt. (g.)	Ubiquinone (μg./g.)	Ubiquinone (μg./organ)	Ubichromenol (μg./g.)	α-Tocopherol (μg./g.)
(A) Basal + B_1 and B_2	172	0·762	137	104	20	27
(B) B_1-deficient	92	0·489	139	68	20	27
(C) B_1-deficient, dosed with B_1	111	0·603	131	79	21	26
(D) B_2-deficient	118	0·575	134	77	22	27
(E) B_2-deficient, dosed with B_2	127	0·624	130	81	22	27

Table 2. *Effect of thiamine and riboflavin on ubiquinone and ubichromenol in rat liver*

Group and treatment	Mean organ wt. (g.)	Ubiquinone (μg./g.)	Ubiquinone (μg./organ)	Ubichromenol (μg./g.)	α-Tocopherol (μg./g.)
(A) Basal + B_1 and B_2	8·77	124	1089	34	41
(B) B_1-deficient	4·71	208	996	55	48
(C) B_1-deficient, dosed with B_1	7·64	108	846	34	25
(D) B_2-deficient	6·27	98	773	26	22
(E) B_2-deficient, dosed with B_2	7·86	95	750	27	22

Table 3. *Effect of thiamine and riboflavin on ubiquinone and ubichromenol in rat spleen*

Group and treatment	Mean organ wt. (g.)	Ubiquinone (μg./g.)	Ubiquinone (μg./organ)	Ubichromenol (μg./g.)	α-Tocopherol (μg./g.)
(A) Basal + B_1 and B_2	0·927	15	14	17	16
(B) B_1-deficient	0·680	17	12	15	17
(C) B_1-deficient, dosed with B_1	0·969	15	14	16	15
(D) B_2-deficient	1·16	13	16	24	13
(E) B_2-deficient, dosed with B_2	0·980	15	15	22	15

whole organ showed little alteration (compare heart, above). The results in spleen show little influence of either of the two vitamins. It seems that neither thiamine nor riboflavin has any specific effect on ubiquinone or ubichromenol concentrations in the rat. What changes there are appear to be produced as a secondary manifestation of the other severe changes occurring during the deficiency states produced by the absence of the two vitamins. It is possible that vitamin E fluctuation, also a secondary manifestation, may be indirectly involved.

Table 4 records the results of the first experiment on pantothenic acid deficiency. Here it was observed that the deficiency state produced a marked increase in the concentration of ubiquinone in the liver. Administration of calcium pantothenate before death reversed the increase in ubiquinone, without producing any significant weight change in the organ. The findings are in contrast with those of Aiyer, Sulebele, Rege & Sreenivason (1959), who observed a slight decrease in the ubiquinone of pantothenic acid-deficient rat liver. The effect of pantothenic acid deficiency in heart could not be considered to be significant. A decrease in the mean weight of this organ occurs during the deficiency, amounting to about 20%, and this resulted in a net loss of ubiquinone from the organ. However, there was no response of heart ubiquinone to administration of pantothenic acid.

The results of the experiment on combined pantothenic acid and vitamin E deficiency are given in Tables 5 and 6. To avoid the difficulties introduced in these studies of the vitamin B deficiencies by the weight fluctuations of the organs, brain was investigated, since the weight of this organ remains practically constant under most conditions (no group mean weight in fact departed from the mean of all the groups by more than 3%). Although in this experiment the differences

Table 4. *Effect of pantothenic acid on ubiquinone and ubichromenol in rat liver and heart*

Group and treatment	Organ	Ubiquinone (μg./g.)	Ubichromenol (μg./g.)	α-Tocopherol (μg./g.)	Vitamin A (i.u./g.)
(F) Supplemented controls	Liver	83	54	28	28
	Heart	157	6	32	1·3
(G) Deficient in pantothenic acid	Liver	146	35	35	35
	Heart	163	10	34	3·0
(H) Deficient in pantothenic acid Dosed with pantothenic acid	Liver	108	42	42	28
	Heart	155	9	30	1·3

Table 5. *Effect of pantothenic acid and vitamin E on ubiquinone and ubichromenol in rat liver*

PA, Pantothenic acid.

Group and treatment	Mean organ wt. (g.)	Ubiquinone (μg./g.)	Ubiquinone (μg./organ)	Ubichromenol (μg./g.)	α-Tocopherol (μg./g.)	Vitamin A (i.u./g.)
(J) Deficient in PA and E	13·7	41	560	14	1·4	65
(K) Deficient in PA and E, dosed PA	12·9	47	610	24	1·3	90
(L) Deficient in PA, dosed E	13·1	55	710	27	11·8	120
(M) Deficient in PA and E, dosed PA and E	13·3	67	880	23	12·6	125
(N) Deficient in E	14·0	33	740	18	1·6	95
(O) Deficient in PA	12·4	60	740	25	22·7	120
(P) Not deficient	14·7	44	640	11	18·1	104
(Q) Deficient in PA with high E	13·1	60	780	26	76·0	150

Table 6. *Effect of pantothenic acid and vitamin E on ubiquinone and ubichromenol in rat brain*

PA, Pantothenic acid; tr., trace; n.d., not detected.

Group and treatment	Mean organ wt. (g.)	Ubiquinone (μg./g.)	Ubichromenol (μg./g.)	α-Tocopherol (μg./g.)	Vitamin A (i.u./g.)
(J) Deficient in PA and E	1·86	28	tr.	5·6	0·4
(K) Deficient in PA and E, dosed E	1·78	27	tr.	5·5	1·2
(L) Deficient in PA and E, dosed PA	1·87	27	tr.	6·9	0·8
(M) Deficient in PA and E, dosed PA and E	1·80	28	tr.	8·4	1·8
(N) Deficient in E	1·85	13	tr.	4·1	n.d.
(O) Deficient in PA	1·84	33	tr.	10·8	1·8
(P) Not deficient	1·85	40	tr.	11·0	1·7
(Q) Deficient in PA with high E	1·82	38	tr.	15·5	1·8

between groups were rather small, the effect of both pantothenic acid and vitamin E in liver confirmed previous findings with these two substances. Although one or two anomalies can be observed, it seems the two vitamins work in opposite senses, pantothenic acid depressing and vitamin E elevating the concentrations of ubiquinone and ubichromenol. The effects in brain, in general, supported these conclusions. Gray, Chisholm & Lee Peng (1960) have recently shown that synthesis of coenzyme A by rat liver is increased by dietary supplementation with tocopherol. In both the present experiments involving single pantothenic acid deficiency (see results for groups G and O) tocopherol concentrations in the liver were increased to some extent, probably significantly. If, as Gray *et al.* suppose, tocopherol is an intermediate in coenzyme A biosynthesis, then pantothenic acid deficiency may lead to a certain sparing action on liver vitamin E. This would be in accordance with the observed increases in liver ubiquinone.

SUMMARY

1. Groups of male and female rats were maintained on thiamine-deficient and riboflavin-deficient diets for 38 days, after which they were killed and their hearts, livers and spleens were analysed for ubiquinone, ubichromenol and α-tocopherol. The concentrations were compared with those found in tissues from control animals, supplemented with the required vitamin. Although certain changes occurred in heart and liver, there appear to be secondary manifestations of severe weight changes during deficiency. Administration of thiamine and riboflavin did not appear to influence ubiquinone or ubichromenol concentrations directly.

2. In pantothenic acid deficiency, groups of rats showed elevated ubiquinone concentrations in liver, but not in heart. A secondary effect, through vitamin E, may be involved.

We wish to thank Mrs W. S. Miller for technical assistance.

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Studies on Vitamin E

8. VITAMIN E, UBIQUINONE AND UBICHROMENOL IN THE RABBIT

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It has been shown by Edwin, Diplock, Bunyan & Green (1961) that vitamin E has a marked effect on the concentrations of ubiquinone and ubichromenol in the tissues of the rat and it was suggested that a biosynthetic relationship existed between the three substances. The rat is an unusual mammal with respect to ubiquinone in that most of the quinone of its tissues consists of ubiquinone 45 (Rüegg *et al.* 1959), whereas the ubiquinone in other mammals so far studied has been found to be ubiquinone 50 (Lester & Crane, 1959). It was therefore of some interest to study the effect of vitamin E in another species, and the rabbit was used as an experimental animal. Edwin *et al.* (1961) drew attention to the significant response of the uterus in the rat to tocopherol administration, compared with that of skeletal muscle, and showed that, in young females, a rapid increase in the tocopherol content of the uterus after dosage resulted in a marked rise in the ubiquinone concentration. It was suggested that these findings might be related to the sensitivity of the female rat to reproductive failure in vitamin E deficiency, and, as a corollary, it was speculated whether the specific symptom of vitamin E deficiency in other species could be correlated with local deficiencies of tocopherol in the tissues. From this point of view the rabbit provides a useful contrast to the rat, for in this species, gestation-resorption does not occur, but the chief deficiency syndrome is a marked muscular dystrophy, which is fatal if untreated. The purpose of this study therefore was to investigate the

distribution of vitamin E in the rabbit, the response of some of the more important tissues to administration of tocopherol and the relationship, if any, between vitamin E and ubiquinone.

EXPERIMENTAL

Animals. Sixteen mature cross-bred female rabbits were purchased from a commercial supplier.

Stock diet. This had the following composition (%): soya-bean meal, 18; fine linseed, 4; grass meal, 42; wheat bran, 14; barley meal, 13; salt, 5; oats, 4.

Vitamin E-deficient diet. This had the following composition: casein (Genatosan Ltd., 'low-vitamin content'), 15%; sucrose, 36·3%; maize starch, 36%; lard, 3%; cod-liver oil, 6%; McCollum's salt mixture, 3%. The following were added to each kg. of diet: choline tartrate, 1 g.; inositol, 1 g.; nicotinic acid, 200 mg.; pyridoxine, 5 mg.; thiamine, 5 mg.; riboflavin, 5 mg.; calcium pantothenate, 10 mg.; folic acid, 5 mg.; menaphthone, 250 μg.; vitamin B_{12}, 70 μg.

Experimental plan. The rabbits were divided into four equal groups. Group A was fed with the stock diet and group B with the deficient diet. Group C was given the deficient diet and in addition received oral doses of α-tocopheryl acetate equivalent to 2 mg./kg. body wt./day for 49 days, and after that until the end of the experiment 4 mg./kg./day. The animals in group D were given the deficient diet and were each given two oral doses of 50 mg. of α-tocopheryl acetate 48 and 24 hr. before they were killed. The experiment lasted 15 weeks. The rabbits in groups B, C and D were examined continuously for signs of muscular dystrophy and their urine was examined weekly for signs of creatinuria. One rabbit in group B displayed the symptoms of dystrophy and creatinuria before the end of the experiment. It was killed, dissected at once and the organs were stored at $-20°$ until the end of the experiment. Two rabbits in group D also showed signs of dystrophy before the end of the experiment, and they were given their two doses of vitamin E and then killed; their organs were stored at $-20°$ until the end of the experiment. After 15 weeks, all the remaining rabbits were killed by cervical dislocation and the required tissues taken. Those from each group were pooled and analysed as described previously by Edwin *et al.* (1961).

RESULTS AND DISCUSSION

The 15-week experimental period is considerably longer than that normally used to produce muscular dystrophy in the rabbit. This was necessary since we particularly wanted to examine the uterus and mature females had to be used. The starting weights of our animals therefore were higher than usual, and not all the deficient animals showed clinical signs of dystrophy or creatinuria even after 15 weeks. The analyses from seven tissues are recorded in Table 1.

The pattern of vitamin E distribution in the rabbits on the stock diet showed certain differences from that observed previously in the rat. Nerve, heart and adipose tissue contained as much tocopherol as the corresponding tissues in the rat, but uterus and skeletal (hind-leg) muscle contained much less. It is clear that the concentrations of tocopherol in the tissues of the rabbit are influenced by the character of the diet (as they are in the rat). Thus the concentration in heart was much lower and in liver much higher in group C animals than in group A animals.

Two significant observations emerge from the analyses of tissues from group B animals. First, although these animals were given the deficient diet for a long time (one was killed *in extremis*) their body fat apparently still contained large amounts of tocopherol. Secondly, skeletal muscle from this group contained one of the lowest concentrations of tocopherol so far observed by us in any animal tissue (0·9 μg./g.). The administration of 100 mg. of α-tocopheryl acetate to deficient animals (group D) seemed to produce a rather smaller effect in terms of increased tissue concentrations than a dose of 10 mg. did in rats (the dosage being approximately equivalent on a body-weight basis), although, in both uterus and skeletal muscle, tocopherol concentrations approximating to those in group A animals were reached. The remarkable concentrations of tocopherol in nerve, and the avidity with which this tissue retains tocopherol during dietary depletion, resembles closely the behaviour of nerve tissue in the rat, discussed by Edwin *et al.* (1961).

Ubiquinone and ubichromenol concentrations in rabbit tissues also showed differences from those in the rat. The concentration of ubiquinone in heart was considerably higher than in any other tissue and was of the same order as that found in rat heart. The ubiquinone concentrations in most of the other rabbit tissues, though, particularly in liver, were generally considerably lower than in the rat. The adipose tissue in the rabbit, however, contained more ubiquinone than was observed in the rat. Ubichromenol was found in every tissue except fat and skeletal muscle from animals in groups A, B and D.

The influence of vitamin E on concentrations of ubiquinone and ubichromenol in the rabbit paralleled that already observed in the rat, but the changes took place at a lower concentration. Comparing tissues from groups B and C, for example, with the exception of brain, in which there was little change, supplementation with tocopherol increased the concentration of ubiquinone and usually increased that of ubichromenol. If the previous estimates of variance made by Edwin *et al.* (1961) in their studies on the rat can be taken as a guide, it would seem that the changes in heart, liver and muscle are significant. Similarly, comparing group B with group A, ubiquinone

concentrations are considerably higher in the heart, liver, uterus and muscle of the group A animals, there being little difference in nerve, brain or fat. Administration of tocopherol to group D animals 48 hr. before they were killed restored the ubiquinone to normal and usually lowered the ubichromenol concentrations in the tissues examined. Again, from our previous estimates, it seems that the differences in heart, liver, uterus and muscle are significant. These observations support the idea that there is a biosynthetic relationship between the three substances in the rabbit as well as in the rat.

A study of the data suggests that the rabbit appears to have more difficulty in the mobilization of tocopherol than does the rat. For example, the heavy concentration of tocopherol in the fat of animals in the deficiency state is striking, and supports the suggestion of Edwin *et al.* (1961) that the clinical nature of the vitamin E-deficiency syndrome, which varies from species to species, might be related to local deficiencies of the vitamin. In confirmation of this, the concentration of α-tocopherol in rabbit muscle is only about one-third of that in rat muscle, and, on depletion, exceptionally low concentrations of tocopherol are produced in the former tissue. Also, the rabbit uterus, unlike the rat uterus, does not contain high concentrations of tocopherol. This again reflects the remarkable differences in the nature of the deficiency syndrome in the two species.

A general conclusion from a study of both species can now be made. It appears that local tissue deficiencies of vitamin E may occur in animals (even to the point of causing death), although no absolute state of deficiency exists in the organism as a whole. It follows therefore that the analyses of tissues that may be quite unrelated to the clinical picture of deficiency (such as, for example, the commonly used serum or liver analyses) are only of limited importance in contributing towards an understanding of the role of vitamin E. It is already fairly clear that neither the total amount of tocopherol in the animal organism nor a calculation

Table 1. *Distribution of vitamin E, vitamin A, ubiquinone and ubichromenol in the female rabbit*

Four groups of four rabbits were used. Group A was on the stock diet, group B on the vitamin E-deficient diet and group C on the supplemented diet. Group D was given the deficient diet and each animal in this group received 100 mg. of α-tocopheryl acetate 48 hr. before death. Average rabbit weights in each group at death were: group A, 2·565 kg.; group B, 2·565 kg.; group C, 2·518 kg.; group D, 2·585 kg. n.d., Not detected.

Tissue	Group	Mean organ wt. (g.)	Ubiquinone (μg./g.)	Ubichromenol (μg./g.)	α-Tocopherol* (μg./g.)	Vitamin A (i.u./g.)
Heart	A	5·83	123	22·6	35·2	2·5
	B	6·34	59	13·5	6·6	1·5
	C	7·39	71	15·3	10·5	1·8
	D	5·82	93	10·0	9·5	2·5
Liver	A	67·2	27·7	8·9	12·1	180
	B	84·7	12·3	6·0	6·9	88
	C	73·9	22·7	7·5	34·5	125
	D	64·5	30·1	4·0	24·3	93
Uterus	A	18·8	12·5	4·4	8·5	1·8
	B	13·9	5·6	1·5	3·0	1·1
	C	19·4	6·0	3·4	3·6	1·3
	D	14·8	7·3	1·3	7·0	1·4
Nerve	A	0·470	12·1	10·0	41·8	7·9
	B	0·426	12·9	10·8	41·5	8·1
	C	0·405	14·0	5·3	50·0	8·7
	D	0·548†	—	—	—	—
Brain	A	7·28	9·5	2·8	12·5	0·9
	B	8·51	8·3	4·3	7·6	0·6
	C	8·80	8·2	3·1	8·5	2·6
	D	7·86	10·2	3·6	8·7	0·9
Skeletal muscle (hind leg)	A	—	13·9	n.d.	4·0	0·8
	B	—	7·2	n.d.	0·9	0·5
	C	—	9·3	3·4	2·7	0·4
	D	—	12·4	n.d.	2·9	0·4
Fat	A	—	14·5	n.d.	120	7·8
	B	—	14·5	n.d.	63·8	4·0
	C	—	30·8	4·9	124	8·4
	D	—	12·2	n.d.	31·6	4·0

* In group A, small amounts of other tocopherols were occasionally observed in the tissues. These were measured, but, being small compared with the α-tocopherol found, have not been recorded here.

† Analyses lost.

from the amounts ingested in the diet are sufficient criteria on which to base judgment of vitamin E status. Much more must be discovered about the actual concentrations of tocopherol in the specific affected sites of vitamin E deficiency in animals, particularly in conditions of dietary or metabolic stress.

SUMMARY

1. Four groups of female rabbits were maintained on various diets in order to study the distribution of vitamin E in their tissues. After a test period of 15 weeks, seven tissues were analysed for tocopherol, vitamin A, ubiquinone and ubichromenol.

2. Skeletal muscle in the rabbit contained little tocopherol and became depleted to exceptionally low levels. This has been related to the muscular dystrophy that occurs in this species. In contrast with the rat uterus, the uterus in the rabbit is little affected by tocopherol. This reflects the difference in the vitamin E-deficiency syndrome in the two species.

3. Vitamin E-deficient rabbits have lower concentrations of ubiquinone in heart, liver and skeletal muscle than animals on the same diet supplemented with α-tocopheryl acetate. In most tissues except nerve and brain, ubichromenol was also lower. Administration of single doses of vitamin E to deficient animals increased ubiquinone in all tissues examined except fat and also decreased ubichromenol. There appears to be a relationship between α-tocopherol, ubiquinone and ubichromenol in the tissues of the rabbit.

4. Rabbits kept on a vitamin E-deficient diet for 15 weeks still contain large reserves of vitamin E in their adipose tissue. Deficiency states can apparently be produced by local deprivation of tocopherol from a specific tissue.

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O-Sulphate Esters of L-Serine, L-Threonine and L-Hydroxyproline

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The hydroxy amino acids, with the exception of tyrosine, have not so far been shown to occur naturally as *O*-sulphate conjugates. The presence of tyrosine *O*-sulphate in one of the two peptides which are liberated from bovine fibrinogen by the action of thrombin was first noted by Bettelheim (1954). This ester has also been detected in similar peptides prepared from rabbit and pig fibrinogens (Blombäck, Boström & Vestermark, 1960; Blombäck & Sjöquist, 1960) and in human and horse fibrins (Bettelheim-Jevons, 1958). These findings raise the possibility that other hydroxy amino acids may occur in proteins as *O*-sulphate conjugates. The wide distribution of the *O*-phosphate conjugates of serine and threonine in phosphoproteins is well known (see Perlmann, 1955).

Attempts to detect *O*-sulphate esters of hydroxylated amino acids in tissues have been preceded by the study of the preparation and properties of the *O*-sulphate esters of L-serine, L-threonine and L-hydroxyproline which is reported here.

EXPERIMENTAL

Preparation of amino acid O-sulphates

Potassium L-serine O-sulphate. The *O*-sulphate esters of tyrosine and tyrosine derivatives have been prepared by Dodgson, Rose & Tudball (1959) with relatively mild conditions of sulphation. A similar method gave a poor yield of potassium L-serine *O*-sulphate. The method of Reitz, Ferrel, Fraenkel-Conrat & Olcott (1946) for the preparation of *O*-DL-serine acid sulphate, when used with L-serine, yielded a product which was contaminated with inorganic SO_4^{2-} ions and unchanged starting material. A satisfactory product was obtained by modifying this method.

L-Serine (1 g.) was added at room temperature to 5 ml. of H_2SO_4 (sp.gr. 1·84) and the mixture stirred until the material dissolved (usually 10 min.). The reaction mixture was kept *in vacuo* for the remainder of 1 hr., when it was poured into 400 ml. of dry ether at −17°. After 15 min. at this temperature the supernatant was decanted and the residue washed (×3) with 200 ml. portions of ether at −17°, the supernatant being decanted after each washing.

The sticky white residue was dissolved in 100 ml. of ice-cold water and inorganic SO_4^{2-} ions were removed by adding an ice-cold saturated solution of $Ba(OH)_2,8H_2O$ until the solution was slightly alkaline. Precipitated $BaSO_4$ was removed by centrifuging and the excess of Ba^{2+} ion in the clear supernatant was precipitated as $BaCO_3$ by passing CO_2 through the cold solution. The whole was clarified by centrifuging and concentrated to dryness *in vacuo* at 38°. The residue was extracted with 20 ml. of ice-cold water and, after centrifuging, the clear extract was passed through a column (7 cm. × 1 cm.) of Dowex 50 ion-exchange resin (Dow Chemical Co., Michigan, U.S.A.; 20–50 mesh; H^+ form). The column was washed with 20 ml. of water and the combined eluate and washings were adjusted to pH 7·8 with aqueous 5% (w/v) KOH before concentrating to dryness *in vacuo* at 38°. The residue was dissolved in the minimum amount of cold water and precipitated by the dropwise addition of ice-cold ethanol. The white crystals were separated at the pump, washed well with ethanol followed by ether and dried *in vacuo* over $CaCl_2$. Yield, 810 mg.; $[\alpha]_D^{16} - 9{\cdot}5°$ in water (*c*, 5·0); $[\alpha]_D^{16} + 8{\cdot}7$ in N-HCl (*c*, 3·4) (Found: ester SO_4^{2-} ion, 40·9; amino N (Van Slyke), 6·1; amino N (formol titration), 6·0; loss at 110° *in vacuo*, 3·7%. $C_3H_6O_6NSK$, $\frac{1}{2}H_2O$ requires ester SO_4^{2-} ion, 41·4; amino N, 6·0; H_2O, 3·9%).

Potassium L-*threonine* O-*sulphate*. This preparation was identical with that for potassium L-serine *O*-sulphate except that after keeping for 1 hr. the reaction mixture was poured into 400 ml. of butan-2-one. The final product was precipitated from aqueous solution with ether–butan-2-one (1:1). Before drying, the crystals were washed with butan-2-one followed by ether. Yield, 0·85 g.; $[\alpha]_D^{18} - 20{\cdot}6$ in water (*c*, 9·7) (Found: ester SO_4^{2-} ion, 38·8; amino N (Van Slyke), 5·4; amino N (formol titration), 5·1; loss at 110° *in vacuo*, 7·1%. $C_4H_8O_6NSK,H_2O$ requires ester SO_4^{2-} ion, 37·6; amino N, 5·5; H_2O, 7·1%).

Potassium L-*hydroxyproline* O-*sulphate*. This preparation was identical with that of L-serine except that the reaction mixture was poured into 300 ml. of crushed ice and water containing 50 g. of $Ba(OH)_2,8H_2O$ rather than into ether. The final product was crystallized by dropwise addition of ethanol–ether (1:1, v/v). Yield, 0·6 g.; $[\alpha]_D^{18} - 26{\cdot}1°$ in water (*c*, 10·5); $[\alpha]_D^{18} - 12°$ in N-HCl (*c*, 9·8) (Found: ester SO_4^{2-} ion, 39·7; imino N (formol titration), 5·2%. $C_5H_8O_6NSK$ requires ester SO_4^{2-} ion, 38·5; imino N, 5·6%).

It is clear from the analytical results that *O*-sulphation rather than *N*-sulphation has occurred. Formol and Van Slyke determinations indicate the presence of free amino (or imino) groupings; moreover, all the esters reacted with ninhydrin and isatin. With the L-serine *O*-sulphate preparation no reaction occurred on treatment of the ester with periodate according to the procedure of Frisell & Mackenzie (1958), suggesting that the terminal hydroxyl group was blocked. Further evidence that *O*-sulphation had occurred in all cases was provided by infrared spectroscopy (see below).

From each amino acid, the same sulphated product was obtained by either the method described or the method of Dodgson *et al.* (1959), although the latter gave lower yields. During the course of many preparations of potassium L-serine *O*-sulphate it has been noted that the water content of the crystals can vary and, in some instances, is absent. In this respect the ester resembles the *O*-phosphate ester of L-serine, which is also known to crystallize in different hydrated forms (cf. Fölsch & Melander, 1957).

Paper chromatography. The homogeneity of all the preparations was checked by descending paper chromatography on Whatman no. 1 with the following solvent systems: I, butan-1-ol–acetic acid–water (50:12:25); II, 2-methylpropan-2-ol–formic acid–water (8:3:4); III, propan-1-ol–ammonia (25% soln.)–water (6:3:1). Chromatograms were usually run overnight and were subsequently detected by spraying with a 0·1% solution of ninhydrin in acetone or, with a 0·2% solution of isatin in acetone, followed by heating at 85° for 5 min. Alternatively, ester sulphate groupings were detected by the method of Lloyd (1960). All the preparations ran as single spots, the areas staining with ninhydrin or isatin coinciding exactly with those giving a positive reaction for ester sulphate. Table 1 shows the chromatographic mobilities of the various esters and parent compounds in the three solvent systems.

Paper electrophoresis. All the esters ran as single homogeneous spots when subjected to horizontal paper electrophoresis on Whatman no. 1 paper in 0·1M-ammonium acetate–acetic acid buffers (ranging from pH 4·0 to 7·0). Electrophoresis was for 2 hr. at a potential of 12 v/cm. Materials were located on the strips as described in the preceding section.

Infrared spectroscopy. The spectra of the esters were determined with the Perkin–Elmer Infracord recording spectrophotometer, the esters being examined as mulls in Nujol (liquid paraffin). The distribution of spectral bands confirmed that the two methods of preparation yielded the same product. Comparison of the spectra with those of a series of alkyl *O*-sulphate esters of known structure provided additional evidence that the amino acids were *O*-sulphated.

X-ray diffraction. X-ray-diffraction patterns of the crystalline powders were kindly prepared by Mr B. Delf of the Department of Physics and confirmed the common identities of the products of the two methods of preparation.

Acid and alkaline hydrolysis

The rates of acid and alkaline hydrolysis of the amino acid *O*-sulphates were determined by estimating the liberated inorganic SO_4^{2-} ions. Weighed samples (20–30 mg.) of the esters were dissolved in 20 ml. of 4% (w/v) trichloroacetic acid (acid hydrolysis) or 20 ml. of N-NaOH (alkaline

Table 1. *Chromatographic mobilities of* L-*serine*, L-*threonine*, L-*hydroxyproline and their corresponding* O-*sulphate esters*

Solvent systems: I, butan-1-ol–acetic acid–water (50:12:25); II, 2-methylpropan-2-ol–formic acid–water (8:3:4); III, propan-1-ol–ammonia (25% soln.)–water (6:3:1). Experimental details are given in the text.

Compound	R_F Solvent I	Solvent II	Solvent III
L-Serine	0·20	0·60	0·51
L-Serine *O*-sulphate	0·12	0·46	0·47
L-Threonine	0·25	0·65	0·57
L-Threonine *O*-sulphate	0·15	0·52	0·49
L-Hydroxyproline	0·25	0·63	0·53
L-Hydroxyproline *O*-sulphate	0·15	0·50	0·49

hydrolysis). Portions (1·5 ml.) were transferred to Pyrex tubes (15 cm. × 1·5 cm.) which were then sealed and placed in an oven at 135° (acid hydrolysis) or, except when otherwise stated, at 100° (alkaline hydrolysis). Zero time was taken as being 2 min. after the tubes were placed in the oven. Tubes were removed from the oven at suitable time intervals and were placed immediately in the deep-freeze (−17°). The cooled tubes were shaken before opening, and samples (0·2 ml.) were withdrawn for determination of inorganic SO_4^{2-} ions by the method of Dodgson (1961*a*, method A).

Enzyme experiments

Biosynthesis of amino acid O-sulphates. Spencer (1960) indicated that free L-serine, L-threonine and L-hydroxyproline were not sulphated when added, at a concentration of 0·01 M and at pH 7·4, to a rat-liver preparation that was capable of sulphating simple alcohols and other hydroxylated compounds. Experimental conditions of pH and acceptor concentration were varied in further attempts to achieve enzymic sulphation of the three amino acids.

A particle-free supernatant fraction (30 μl.) of a rat-liver suspension, prepared according to the directions of Spencer (1960), was incubated for 1 hr. at 38° with 10 μl. of an aqueous solution of the amino acid (adjusted to the appropriate pH with a trace of 0·05 N-NaOH), 10 μc (approx. 1 μl.) of $Na_2{}^{35}SO_4$ (code SJS 1, The Radiochemical Centre, Amersham, Bucks) and 10 μl. of a solution containing 1 μmole of adenosine triphosphate, 1 μmole of KH_2PO_4 and 0·3 μmole of $MgCl_2$, the solution being adjusted to the appropriate pH with N-NaOH. Control experiments were made, in which water was substituted for the amino acid solution. After incubation the reaction tubes were placed in a boiling-water bath for 2 min. before centrifuging. Samples (10 μl.) of the clear supernatants were subjected to descending paper chromatography on Whatman no. 1 paper with solvent systems I and II. After drying, radioautographs were prepared by placing the papers in contact with Ilford Industrial B X-ray film for 7 days. The appearance on the test radioautographs of a new radioactive spot in the position which the particular amino acid *O*-sulphate would be expected to occupy was considered to indicate enzymic sulphation.

Enzymic desulphation. Limited searches (e.g. Neuberg & Wagner, 1925; Roy, 1953; Dodgson, 1961*b*) have failed to detect the presence, in Nature, of an enzyme capable of desulphating relatively simple alkyl sulphate esters. Although it therefore seemed unlikely that the three amino acid *O*-sulphates would undergo enzymic desulphation by tissue preparations, the possibility was checked as follows. Aqueous suspensions (4%, w/v) of rat tissues were prepared with a glass homogenizer. A portion (0·1 ml.) of the suspension was incubated at 38° for 18 hr. with an equal volume of a 0·02 M solution of each amino acid *O*-sulphate in 0·25 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–acetic acid buffer, pH 6·0, 7·0 and 8·0. After incubation, 0·8 ml. of ethanol was added and liberated sulphate was determined by the chloranilate procedure of Lloyd (1959). Control determinations were made in which enzyme and substrate were incubated separately and mixed only just before adding the ethanol. Preliminary experiments showed that inorganic SO_4^{2-} ions (K_2SO_4) could be recovered quantitatively under these conditions.

RESULTS

Acid and alkaline hydrolysis

Figs. 1 and 2 show the rates of liberation of inorganic SO_4^{2-} ions from the three amino acid *O*-sulphates. The stability of the three esters under

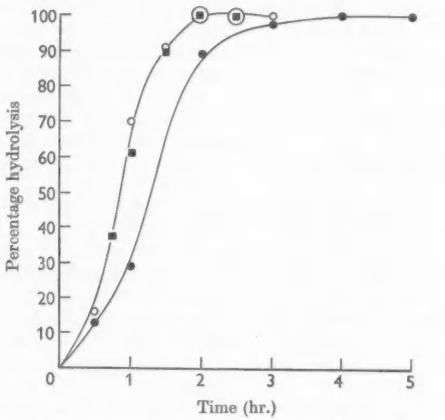

Fig. 1. Rates of hydrolysis of: ●, L-serine *O*-sulphate (5 mM); ○, L-threonine *O*-sulphate (4 mM); ■, L-hydroxyproline *O*-sulphate (5·8 mM), by 4% (w/v) trichloroacetic acid at 135°. Experimental details are given in the text.

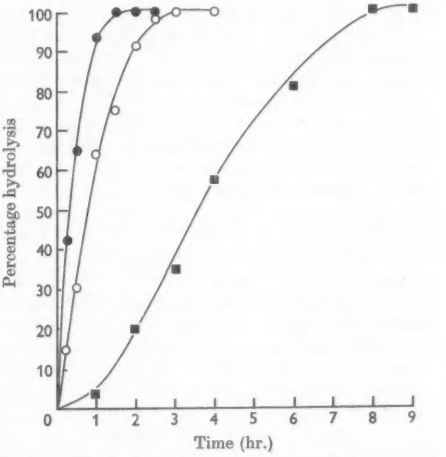

Fig. 2. Rates of hydrolysis of: ●, L-serine *O*-sulphate (4·9 mM); ○, L-threonine *O*-sulphate (3·8 mM); ■, L-hydroxyproline *O*-sulphate (6·5 mM), by N-NaOH. Hydrolysis of L-serine *O*-sulphate and L-threonine *O*-sulphate was carried out at 100°; hydrolysis of L-hydroxyproline *O*-sulphate was at 115°.

acidic conditions is very similar. Under alkaline conditions the *O*-sulphate esters of L-serine and L-threonine are relatively unstable, complete hydrolysis being achieved in 90 and 180 min. respectively. In contrast, L-hydroxyproline *O*-sulphate was relatively stable to alkali. The behaviour of L-serine *O*-sulphate is not unexpected in view of the known alkaline instability of the corresponding *O*-phosphate ester (see Perlmann, 1955). Separate experiments showed that slight release of SO_4^{2-} ion from L-serine *O*-sulphate occurred (approx. 4% in 24 hr.) at room temperature in the presence of N-NaOH. No measurable hydrolysis of the ester occurred under the same conditions in the presence of trichloroacetic acid.

Attempted sulphation of L-*serine,* L-*threonine and* L-*hydroxyproline by rat-liver preparations*

No evidence was obtained that the three amino acids, at final concentrations of 0·01 M and 0·066 M, could undergo sulphation in the presence of rat-liver-supernatant preparations at pH 7·4. All test and control radioautographs showed the presence of many ^{35}S-containing compounds. Spencer (1960) has shown that these compounds are ester sulphates arising from the sulphation of endogenous hydroxylated acceptors. Additional spots did not appear on the test radioautographs when L-serine, L-threonine or L-hydroxyproline was present in the incubation mixtures.

During attempts to achieve the enzymic sulphation of various tyrosine derivatives, Segal & Mologne (1959) showed that, in certain cases, a high pH (9·3) favoured the sulphation. They suggested that suppression of the ionization of the α-amino grouping might be partly responsible for this effect. An attempt was therefore made to sulphate L-serine, L-threonine and L-hydroxyproline with liver preparations at pH 9·3, two concentrations of acceptor being used as before. Negative results were obtained in all cases.

Enzymic desulphation of the amino acid O-*sulphates*

No significant liberation of inorganic SO_4^{2-} ions occurred when the three amino acid *O*-sulphates were incubated with suspensions of rat pancreas, spleen, heart, kidney and lung. With liver preparations some release of SO_4^{2-} ions from L-serine *O*-sulphate was observed. In the most active preparation tested, the release of SO_4^{2-} ions at pH 6·0, 7·0 and 8·0 corresponded respectively to 2, 25 and 15% hydrolysis of the substrate over a period of 18 hr. Liver preparations had no effect on the *O*-sulphates of L-threonine and L-hydroxyproline under similar conditions. A pH of 7·0 was used in all subsequent experiments with L-serine *O*-sulphate.

In attempts to obtain greater enzyme activity a more concentrated preparation was obtained as follows. Fresh rat liver was squeezed through a fine mesh to remove connective tissue, and 13 g. of the resultant pulp was suspended in water at 4° with a glass homogenizer, and made up to 100 ml. with water. The suspension was then alternately frozen and thawed three times and subsequently resuspended with a glass homogenizer. A portion (25 ml.) of the suspension was withdrawn and kept at 4° (fraction 1) and the remainder was dialysed against 150 vol. of water at 4° for 4 hr. After dialysis, the contents of the dialysis bag were resuspended as usual and a portion (25 ml.) was withdrawn and stored at 4° (fraction 2). The remainder was centrifuged at 2500 *g* and 2° for 20 min., and the faintly cloudy supernatant was separated and kept at 4° (fraction 3). The activities of the three fractions towards 0·01 M-L-serine *O*-sulphate in the presence of 0·25 M-tris–acetic acid buffer, pH 7·0, was then determined. The slight increase in volume which occurred as a result of the dialysis procedure was ignored in comparing the activities of the three fractions.

The results suggested that the bulk of the activity which was present in the original suspension (fraction 1) was still present in the final supernatant fraction (fraction 3). However, it became apparent from several experiments that recoveries of inorganic SO_4^{2-} ions from these various fractions were poor and inconsistent. To overcome this difficulty a further series of experiments were made with L-serine *O*-sulphate that had been labelled with ^{35}S. This material was prepared from 0·15 g. of L-serine and 0·5 ml. of $H_2{}^{35}SO_4$ (sp.gr. 1·84; specific activity 2·64 mc/m-mole). The final preparation had a specific activity of approx. 11 μc/mg. and was mixed with unlabelled L-serine *O*-sulphate in the proportions 1:2.

L-Serine *O*-[^{35}S]-sulphate (0·1 ml. of a 0·02 M-solution in 0·5 M-tris–acetic acid buffer, pH 7·0) was incubated at 38° with 0·1 ml. of the rat-liver supernatant (fraction 3) for 1, 2, 8 and 18 hr. At the end of the appropriate incubation period the reaction tubes were placed in boiling water for 2 min. After cooling, coagulated protein was removed by centrifuging and 20 μl. of the clear supernatant was applied to Whatman no. 1 paper (32 cm. × 11 cm.) and subjected to horizontal paper electrophoresis in aqueous 0·1 M-ammonium acetate for 2 hr. at a potential gradient of 12 v/cm. Control determinations were made in which the liver preparation was added to the substrate and the whole immediately placed in a boiling-water bath for 2 min. After cooling, the controls were incubated at 38° for the appropriate period and were then treated exactly as described for the test determinations except that heating to 100° after incubation was omitted. Solutions of L-serine *O*-[^{35}S]sulphate and inorganic

$^{35}SO_4^{2-}$ ion ($Na_2{}^{35}SO_4$) were run on the same paper as markers. The paper sheets were air-dried and cut into appropriate strips (1 in. wide). The radioactive zones were located and the relative radioactivity of these zones was measured with the C. 100 Actigraph automatic strip-scanner (Nuclear–Chicago Corp., Ill., U.S.A.). Strips were scanned at a speed of 12 in./hr. with a slit width of 0·125 in., an integration time of 50 sec. and a scale setting of 1000 counts/min. Fig. 3 shows the relative amounts of liberated inorganic SO_4^{2-} ions and residual L-serine *O*-[^{35}S]sulphate for the various incubation periods. The areas of the various peaks of radioactivity on the scanning chart were used in calculating the approximate rate of liberation of inorganic $^{35}SO_4^{2-}$ ion, which over periods of 1, 2, 8 and 18 hr. corresponded to a percentage hydrolysis of 28·7, 47·4, 54·0 and 61·0 respectively. Although it seemed clear from these experiments that inorganic $^{35}SO_4^{2-}$ ion was being liberated from L-serine *O*-[^{35}S]sulphate, the possibility that the ester was being metabolized so as to give a sulphated product that moved on paper electrophoresis at the same rate as inorganic $^{35}SO_4^{2-}$ ion was eliminated in the following way. L-Serine *O*-[^{35}S]sulphate (40 μl. of a 0·02 M-solution in 0·5 M-tris–acetic acid buffer, pH 7·0) was incubated at 38° for 3 hr. with 40 μl. of the rat-liver preparation (fraction 3). After incubation, the reaction tube was placed in a boiling-water bath for 2 min. A control determination was made as described above. After cooling, precipitated protein was removed by centrifuging, and 60 μl. of the clear supernatant was withdrawn and added to a mixture of 1·5 ml. of aqueous (0·15 M) carrier K_2SO_4, 4 ml. of 2·5 N-HCl and 30 ml. of water. After mixing, inorganic sulphate was precipitated by the dropwise addition of 4 ml. of an aqueous 10 % solution of $BaCl_2$. Precipitated $BaSO_4$ was separated by centrifuging and washed (with intermediate centrifuging) with three 40 ml. portions of water followed by 40 ml. of acetone. After drying at 110° the $BaSO_4$ was plated and radioactivity measured (at infinite thickness) with a thin mica end-window Geiger–Müller tube. Preliminary experiments showed that L-serine *O*-[^{35}S]sulphate was not hydrolysed or co-precipitated with $BaSO_4$ during the acidic precipitation procedure. The radioactivity by the $BaSO_4$ obtained in the test determination was approx. six times as great as that obtained in the control, confirming that enzymic liberation of inorganic $^{35}SO_4^{2-}$ ion had occurred.

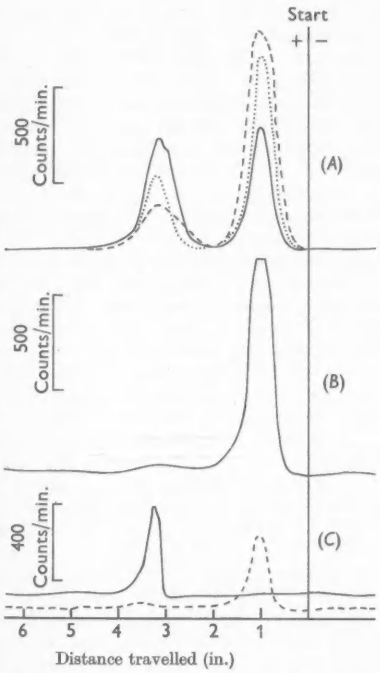

Fig. 3. Continuous scanning records of radioactive zones obtained by paper electrophoresis after incubating L-serine *O*-[^{35}S]sulphate with a rat-liver preparation (fraction 3) in the presence of 0·25 M-tris–acetic acid buffer, pH 7·0, for various periods at 38°. (*A*) Superimposed test determinations: 1 hr. incubation period (broken line); 2 hr. incubation period (dotted line); 8 hr. incubation period (solid line). (*B*) A typical control experiment with boiled enzyme. (*C*) Samples of inorganic [^{35}S]sulphate (solid line) and L-serine *O*-[^{35}S]sulphate (broken line) run separately on the same electrophoresis papers as the incubation mixtures. The scans have been stylized and the curve for the 18 hr. incubation period is omitted for clarity. See text for experimental details.

In a separate experiment the relative enzyme activities of fractions 1, 2 and 3 over an incubation period of 6 hr. were compared as described above. The relative activities of the fractions were similar (Fig. 4) and corresponded to a percentage hydrolysis of 47·0, 46·6 and 48·5 respectively.

The activity of fraction 1 was also checked in the presence of 0·25 M-sodium acetate–acetic acid buffer, pH 7·0, and in the presence of a 0·25 M-KH_2PO_4 solution that had been adjusted to pH 7·0 with 10 % (w/v) NaOH. The enzyme activities of the fraction in the presence of acetate and phosphate over a period of 6 hr. corresponded to a percentage hydrolysis of 38·4 and 39·6 respectively.

Fig. 5 shows the effect of pH on the rate of liberation of inorganic $^{35}SO_4^{2-}$ ion from 0·01 M-L-serine *O*-[^{35}S]sulphate in the presence of 0·25 M-tris–acetic acid buffer. A preparation of fraction 3

was used as the enzyme source, incubation was for 3 hr. at 38°, and the liberated inorganic $^{35}SO_4^{2-}$ ion was determined as before.

DISCUSSION

Previous work with L-tyrosine and related derivatives (Dodgson *et al.* 1959) has shown that relatively mild treatment with sulphuric acid results in the sulphation of the hydroxyl group and does not affect the amino grouping or the benzene ring. More drastic conditions of sulphation can give rise to ring sulphonates, and sulphation with chlorosulphonic acid leads to the formation of sulphamates. Reitz *et al.* (1946) have reported that mild treatment of proteins with sulphuric acid leads to the sulphation of hydroxylated amino acids, but prolonged treatment leads to some pronounced change which is accompanied by sulphate elimination. This could be interpreted by postulating a N → O shift. Elliot (1952) showed that sulphuric acid caused this type of shift about the serine and threonine residues in silk fibroin and lysozyme, and similar findings have been made for the serine residues of poly-DL-serine (Fasman, 1960). In the present work, the mild treatment in the method of Dodgson *et al.* (1959) and the relatively severe treatment in the method described both yielded *O*-sulphate esters only.

The present work confirms and extends the findings of Spencer (1960) that free L-serine, L-threonine and L-hydroxyproline do not undergo sulphation by fortified liver-supernatant preparations which are able to sulphate other hydroxylated compounds. A number of workers have made similar findings with L-tyrosine (see Dodgson, 1958), and Segal & Mologne (1959) have produced evidence which indicates that, before L-tyrosine can be enzymically sulphated, the carboxyl group must be blocked and the amino group present in an undissociated form. Similar conditions may well apply to the enzymic sulphation of L-serine, L-threonine and L-hydroxyproline, and the negative results reported here should not necessarily be taken as indicating that L-serine *O*-sulphate cannot be present in proteins.

The findings that rat liver contains an enzyme system capable of achieving the desulphation of the *O*-sulphate ester of L-serine but not the corresponding esters of L-threonine and L-hydroxyproline, raises some interesting points. The presence of an enzymic mechanism for the desulphation of

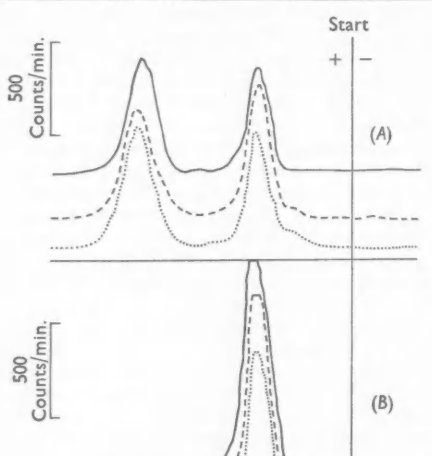

Fig. 4. Continuous scanning records of radioactive zones obtained by paper electrophoresis after incubating L-serine *O*-[^{35}S]sulphate with rat-liver fractions 1–3 (see text for details). Incubation was for 6 hr. at 38° in the presence of 0·25 M-tris–acetic acid, pH 7·0. (*A*) Test determinations: fraction 1 (dotted line); fraction 2 (broken line); fraction 3 (solid line). (*B*) Control determinations (designated as for test determinations). The curves have been stylized and displaced vertically to facilitate comparison.

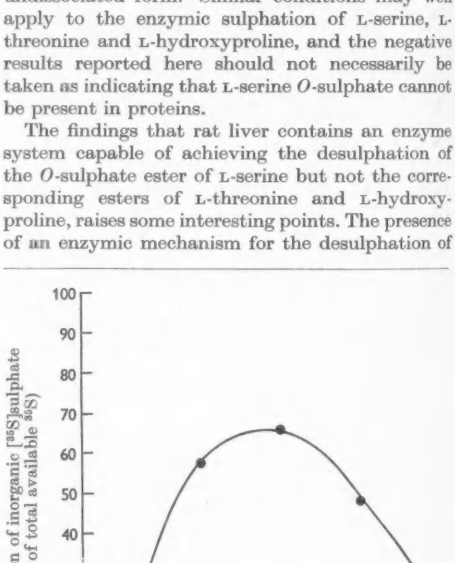

Fig. 5. Effect of pH on the liberation of inorganic [^{35}S]-sulphate from 0·01 M-L-serine *O*-[^{35}S]sulphate by a rat-liver preparation (fraction 3). Incubation was for 3 hr. at 38° in 0·25 M-tris–acetic acid buffer.

L-serine *O*-sulphate may indicate that this ester is of some significance in metabolism. However, it has not yet been established that a simple hydrolase (a sulphatase) is involved and the appearance of inorganic SO_4^{2-} ion may merely reflect the activity of an entirely different type of enzyme system. For example, the replacement of the α-amino grouping of L-serine *O*-sulphate with a keto grouping by an enzyme system analogous to that responsible for the conversion of 3-phosphoserine into 3-phosphohydroxypyruvate (Ichihara & Greenberg, 1957) would yield the *O*-sulphate ester of 3-hydroxypyruvic acid. Such a compound might well be unstable and tend to lose SO_4^{2-} ion spontaneously. Other similar possibilities could be argued, and under such circumstances the actual release of SO_4^{2-} ion, although in itself a non-enzymic process, would appear to be enzymic in experiments such as those described above. Reaction sequences of this type might be expected to be cofactor-dependent and it must be borne in mind that fraction 3 of the liver preparation used in this work has been dialysed, although for a limited period (4 hr.) only.

A sulphatase could be responsible for the desulphation of L-serine *O*-sulphate although, with one exception (the type-1 arylsulphatases), all the sulphatases known at present are strongly (often completely) inhibited by phosphate. Desulphation of L-serine *O*-sulphate still proceeds at an appreciable rate in the presence of phosphate. Dodgson (1961*b*) has noted that partially purified extracts of the digestive organs of the periwinkle (*Littorina littorea*), which contain arylsulphatase, 3β-steroid sulphatase, glycosulphatase and cortisone 21-sulphatase, are without effect on L-serine *O*-sulphate. The digestive juice of *Helix pomatia* (containing arylsulphatase, 3β-steroid sulphatase and cortisone 21-sulphatase) was also unable to desulphate the ester.

Although the quantitative experiments suggest that the rat-liver enzyme system is only a moderately active one, preliminary experiments (unpublished) have shown that L-serine *O*-[^{35}S]sulphate undergoes appreciable desulphation when injected into rats.

SUMMARY

1. Methods have been described for the preparation of the *O*-sulphate esters of L-serine, L-threonine and L-hydroxyproline.

2. The esters exhibit a similar stability towards acid, but L-serine and L-threonine *O*-sulphates are much less stable to alkali than the corresponding L-hydroxyproline derivative.

3. Enzymic sulphation of the three amino acids by a rat-liver system known to be capable of sulphating other hydroxylated compounds could not be achieved.

4. Rat liver contains an enzyme system, the activity of which results in the liberation of inorganic sulphate from L-serine *O*-sulphate. Maximum enzyme activity is obtained in the region of pH 7·0.

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Acidic Peptides of the Lens

6. METABOLISM OF γ-GLUTAMYL PEPTIDES IN SUBCELLULAR FRACTIONS OF RABBIT LIVER*

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Ophthalmic acid (I; R = CH_3), an analogue of glutathione (I; R = SH), has been isolated from calf lens (Waley, 1958), and probably also occurs in rat liver..

$$\begin{array}{l} H_2N\cdot CH\cdot[CH_2]_2\cdot CO\cdot NH\cdot CH\cdot CO\cdot NH\cdot CH_2\cdot CO_2H \\ \quad\quad | \quad\quad\quad\quad\quad\quad\quad\quad | \\ \quad CO_2H \quad\quad\quad\quad\quad\quad CH_2R \end{array}$$

(I)

The two tripeptides are similar in structure; they share some biological properties (Cliffe & Waley, 1958*b*), and compete in others (Cliffe & Waley, 1959*a*) and are synthesized by the same route in extracts of calf lens (Cliffe & Waley, 1958*a*). The comparison between ophthalmic acid and glutathione has now been extended by the choice of a tissue that not only contains enzymes that bring about the synthesis of the tripeptides but also enzymes that catalyse their breakdown. Such a tissue is rabbit liver.

Subcellular fractionation has been used to study these enzymes. This method enabled us to find out whether both glutathione and ophthalmic acid were synthesized, or broken down, in one fraction (i.e. we compared substrates) and whether the synthesis and breakdown took place in the same fraction.

The results of our comparisons are, in brief, as follows. There is a parallel distribution of the enzymes concerned in the metabolism of glutathione and ophthalmic acid; they may, indeed, be the same enzymes, and, where it has been tested, this idea is supported by the results of competition experiments. Synthesis and breakdown take place (mainly) in different fractions: the nuclear fraction contains the enzyme (γ-glutamyl transpeptidase) that catalyses the hydrolysis of the tripeptides, and the supernatant fraction contains the enzyme system that brings about their synthesis. A preliminary account of this work has already appeared (Cliffe & Waley, 1959*b*).

MATERIALS AND METHODS

Tissues. Lenses were removed from the eyes of calves at the slaughterhouse within 2 hr. after death of the animals. Livers of rabbits (3–5 months old), guinea pigs (adult), pigeons (adult) and rats (90–150 g.) were used fresh from laboratory animals which had been starved for 24 hr. Rabbits and guinea pigs were killed by a sharp blow on the back of the neck, and pigeons and rats by decapitation. Calf liver and sheep kidney were obtained fresh from the slaughterhouse and brought to the laboratory packed in ice.

Dialysed calf-lens preparation. This was prepared as described by Cliffe & Waley (1958*a*) and was sometimes stored at −15° overnight before use.

Peptides and amino acids. Ophthalmic acid, norophthalmic acid, γ-L-glutamyl-L-α-amino-*n*-butyric acid and L-α-amino-*n*-butyrylglycine were prepared as described by Waley (1958); the glutathione was a gift from The Distillers Co. Ltd. (Biochemicals); other peptides and amino acids were commercial preparations.

Solutions of glutathione, and cysteine hydrochloride were prepared immediately before use, and the pH was brought to 6·6 with aqueous KOH. Other solutions of acidic amino acids and peptides were neutralized with aqueous KOH.

Cofactors. The barium salt of D-3-phosphoglyceric acid (L. Light and Co. Ltd., Colnbrook, Bucks.) was converted into the soluble potassium salt with Amberlite IR-120(H^+) and KOH (Wade & Morgan, 1955).

Phosphate buffer–salt medium. This contained potassium phosphate buffer, pH 7·4, (70 mM), KCl (70 mM) and $MgSO_4$ (7 mM).

Liver preparations

All manipulations were done as quickly as possible at 0–2°. The homogenizer, Fischer mincer and all the reagents were chilled before use.

Whole liver. The liver was chilled in ice-cold 0·15M-KCl, blotted lightly and forced through a Fischer mincer ($\frac{1}{16}$ in. mesh grid). The mince was homogenized for 2 min. with phosphate buffer–salt medium (2 ml./g. wet wt. of liver) in a stainless-steel homogenizer of the Potter-Elvehjem type. Preparations of sheep-kidney cortex were made in the same way.

Subcellular particles. The method of fractionation was similar to that of Schneider (1948). Whole liver mince was homogenized for 2 min. with 0·25M-sucrose and made up in boiled water (4 ml./g. wet wt. of mince). Three fractions were prepared from the whole homogenate by differential centrifuging in a refrigerated centrifuge: nuclei and debris (which will be referred to as crude nuclei) were sedimented at 600*g* for 10 min., mitochondria were sedimented at 8500*g* for 10 min., and microsomes were sedimented at 19000*g* for 60 min. The crude nuclei were washed twice by resuspension in 0·25M-sucrose (1 ml./g. wet wt. of mince) and resedimented at 600*g* for 10 min., and the mitochondria were washed in a similar way with 0·25M-sucrose

* Part 5: Waley (1959).

(0·5 ml./g. wet wt. of mince) and resedimented at 8500 *g* for 10 min. At each stage the washings were added to the remaining unfractionated material. The 'fluffy layer', which sedimented immediately on top of the mitochondria, was kept with the mitochondrial fraction. All resuspension of particles was effected with a revolving Perspex pestle machined to fit the centrifuge tubes.

For experiments in which peptide breakdown was assayed, the particles were resuspended in phosphate buffer–salt medium, and the suspension was kept at 4° for up to 4 hr. while the other particles were separated. For experiments in which peptide synthesis was studied, the fractions were suspended in water (about 5 ml.) and an acetone-dried powder was prepared. This was extracted by the procedure of Snoke & Bloch (1955).

The number of intact cells in the crude nuclear fraction was determined. A suspension in 0·25 M-sucrose spotted on six separate microscope slides was examined under the phase-contrast microscope at a magnification of 500 times. The number of nuclei and intact cells were counted in five fields chosen at random on each slide; the concentration of the suspension was adjusted so that the total number of nuclei and intact cells in each microscope field was about 30. Assuming (*a*) that no nuclei were lost during homogenization or fractionation and (*b*) that about 30% of the liver cells were binucleate, the proportion of remaining intact cells was calculated. In the absence of a value for the number of binucleate cells in rabbit liver, the value for rat liver (Harrison, 1953) has been used. The proportion of intact cells in the crude nuclear fraction was consistently in the range 5–10% and was not lowered by homogenization for 4 min. instead of 2 min. On two occasions, the nuclear fraction was prepared from rat liver by the same procedure as described above for rabbit liver, and the percentage of intact cells was 2–5%. The cells of rabbit liver seem more difficult to disrupt than those of rat liver.

Alternative preparation of nuclei. Rabbit liver was perfused *in situ* (immediately after death) via the portal vein with 0·15 M-KCl (0·5–1 l.) followed by 0·25 M-sucrose solution containing 1·8 mM-$CaCl_2$ (40 ml.). The gall bladder was removed and the liver excised. The liver was blotted dry, forced through a Fischer mincer and homogenized for 2 min. in a medium of 0·25 M-sucrose containing 1·8 mM-$CaCl_2$ (9 ml./g. wet wt. of perfused mince). The homogenate was sieved through a layer of sterile surgical lint, and the filtrate was homogenized for a further 2 min. The number of intact cells was now 1–2%. The nuclei were separated from the whole homogenate as described by Hogeboom, Schneider & Striebich (1952). They were washed three times by resuspension in the medium and packed into a small pellet by centrifuging at 10 000 *g* for 10 min. A suspension of nuclei in phosphate buffer–salt medium was used for enzymic studies.

Supernatant fraction. As the enzymic activities that we were concerned with were low they could not be measured in the supernatant fraction unless it was concentrated. A whole liver homogenate was prepared as described above and centrifuged at 19 000 *g* for 60 min. to sediment the particles; the supernatant fraction was removed with a bent-tipped pipette. For experiments in which peptide breakdown was studied, the supernatant fraction was dialysed overnight at 4° against water to remove the sucrose, and an acetone-dried powder prepared. This was extracted at 4° by occasional grinding during 1 hr. with phosphate buffer–salt medium; about 2·5 ml./0·4 g. of powder usually gave a convenient concentration of enzyme for assay. The mixture was centrifuged at 3000 *g* for 10 min. at 1°, and the supernatant was used. For experiments on the synthesis of peptides, the supernatant fraction was dialysed at 4°, first against 0·08 M-$NaHCO_3$, pH 8, for 4 hr., and then against water for a further 12 hr. An acetone-dried powder was prepared and extracted as described by Snoke & Bloch (1955).

Preparation of acetone-dried powders. In general, the tissue or subcellular fraction was macerated with acetone cooled to −10° (10 ml./g. wet wt. or /ml.). The precipitate was collected on a Büchner funnel with a Whatman no. 52 paper, and washed with more cold acetone. The pad of insoluble material was carefully scraped off the filter paper (a process which is easier when the hard no. 52 paper is used) and finally dried in a vacuum desiccator overnight.

Standard reaction mixture

System used for the synthesis of peptides. The reaction mixture contained adenosine triphosphate (ATP) (Sigma Chemical Co.) (4 mM), 3-phosphoglyceric acid (33 mM), KCl (100 mM), $MgSO_4$ (10 mM), KCN (15 mM), 2-amino-2-hydroxymethylpropane-1:3-diol buffer (100 mM), glycine (15 mM), L-glutamic acid (10 mM), L-cysteine (10 mM) or DL-α-amino-*n*-butyric acid (20 mM), and 1 ml. of an extract of acetone-dried whole liver or subcellular particles prepared by the method of Snoke & Bloch (1955), in a total volume of 2 ml. In some experiments 0·16 ml. of dialysed calf lens was included. The mixture was incubated for 6 hr. at 37° and the pH was 7·8. Samples (0·2 ml.) were withdrawn at hourly intervals for estimation.

System used for the breakdown of peptides. The reaction mixture contained ophthalmic acid (4 mM) or glutathione (4 mM) or γ-L-glutamylglycine (10 mM) and 0·45 ml. of liver preparation in phosphate buffer–salt medium in a total volume of 0·6 ml. It was incubated for 5 hr. at 37° and the pH was 7·4; 0·05 ml. samples were withdrawn at 30 min. intervals.

Following the course of the reactions

Removal of protein. Samples from the reaction mixtures were pipetted into centrifuge tubes containing 1 ml. of the appropriate concentration of acetic acid to give a final pH of 5·3 (Hunter, 1957). The tubes were heated in a boiling-water bath for 3 min. with glass 'bobbles' on top, cooled and centrifuged at 3000 *g* for 5 min. A measured portion of supernatant was dried in a vacuum desiccator. The residues were taken up in 10% (v/v) propan-2-ol and examined by paper electrophoresis or paper chromatography.

Removal of glutathione. Reduced glutathione (GSH) has the same electrophoretic mobility as ophthalmic acid and therefore interferes with the estimation of ophthalmic acid on electrophoresis papers. In experiments where both tripeptides were present in the reaction mixtures, glutathione was oxidized to the sulphonic acid (GSO_3H), which has a higher electrophoretic mobility towards the anode at pH 4.

The residues of samples after deproteinization were taken up in performic acid solution, which was freshly prepared on each occasion by mixing 0·1 ml. of H_2O_2 (A.R., 30%, w/v) and 0·9 ml. of formic acid (A.R., 98–100%). Twenty μl. of performic acid/μmole of sulphydryl compound was used;

this is sufficient for the reaction to go to completion. (In calculating the amount of performic acid the cysteine, which was in the samples, was also taken into account.) The solutions were left for 1 hr. and evaporated to dryness in a vacuum desiccator. Excess of performic acid was decomposed by taking up the residue in water and evaporating to dryness again.

Separation and estimation of peptides and amino acids. In most cases the compounds were separated by paper electrophoresis at pH 4 (Cliffe & Waley, 1958*a*). On occasions they were separated by descending paper chromatography on Whatman no. 52 paper with butan-1-ol–acetic acid–water (40:9:20, by vol.) as the solvent system. Glutathione was separated (as GSSG) by descending paper chromatography on Whatman no. 52 paper with 72% (w/w) phenol–3% (w/v) NH_3 soln. as solvent. The electrophoresis and chromatography papers were dried and developed in ninhydrin–$CoCl_2$ reagent, and the intensities of the spots measured by direct photometry on the paper (Cliffe & Waley, 1958*a*).

RESULTS

γ-Glutamyl lactamase

The distribution of this enzyme in subcellular fractions of rabbit liver is described first, as the results are particularly clear-cut. The substrate chosen to assay the enzyme was γ-L-glutamylglycine (γ-Glu.Gly); the reaction catalysed by γ-glutamyl lactamase leads to 5-oxopyrrolidine-2-carboxylic acid (PCA) and glycine (Gly) (Connell & Hanes, 1956):

$$H_2N\cdot CH(CO_2H)\cdot[CH_2]_2\cdot CO\cdot NH\cdot CH_2\cdot CO_2H$$

γ-Glu.Gly

$$\downarrow$$

$$\underbrace{HN\cdot CH(CO_2H)\cdot[CH_2]_2\cdot CO}_{\text{PCA}} + \underbrace{H_2N\cdot CH_2\cdot CO_2H}_{\text{Gly}}$$

The reaction is detected and followed by paper electrophoresis; the spot due to the dipeptide (γ-glutamylglycine) disappears, and only one ninhydrin-positive spot (glycine) appears. The other product of the reaction 5-oxopyrrolidine-2-carboxylic acid was detected with chlorine (Reindel & Hoppe, 1954). Glutamic acid is not formed. The formation of 5-oxopyrrolidine-2-carboxylic acid is characteristic of γ-glutamyl lactamase; this criterion will be used later in discussion of the breakdown of ophthalmic acid.

The breakdown of γ-glutamylglycine, catalysed by an extract of acetone-dried rabbit liver, was first-order (Fig. 1). The rate constant (k) from the plot, in units of min.$^{-1}$, is converted into units of 1 g. wet wt. of liver mince/ml. of incubation mixture (k') in Table 1, which also shows the activities of the subcellular fractions.

The figures in Table 1 were arrived at as follows: the fifth row is taken as an example. Minced liver (3 g.) yielded an acetone powder (0·65 g.), a portion (0·37 g.) of which was extracted with buffer (2·5 ml.). A sample (0·45 ml.) of this extract was used for the reaction; the volume of the reaction mixture was 0·6 ml., and the rate constant (k)

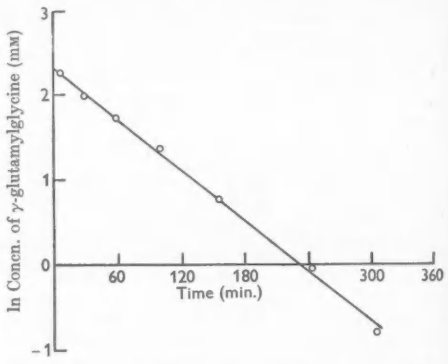

Fig. 1. Breakdown of γ-glutamylglycine, catalysed by an extract of acetone-dried rabbit liver. The products are 5-oxopyrrolidine-2-carboxylic acid and glycine.

Table 1. *Breakdown of γ-glutamylglycine in subcellular fractions of rabbit liver*

The conditions for the assays, and the meaning of k' are given in the text. The relative specific activity of a fraction is derived from the figures in the third and fourth columns, e.g. 85/100 ÷ 118/217·5 = 1·6, and takes into account the differing amounts of protein in each fraction.

	10^3k (min.$^{-1}$)	$10^3k'$	Activity in fraction (% of activity in whole liver)	Amount of protein in each fraction (mg.)	Relative specific activity
Whole liver	11·52	46·1	(100)	217·5	(1·0)
Nuclear fraction	0·99	1·3	3	44·5	0·1
Mitochondria	3·00	1·1	2	23·3	0·2
Microsomes	5·07	2·3	5	30·6	0·4
Whole liver (acetone powder)	9·46	18·4	(100)	217·5	(1·0)
Supernatant fraction (acetone powder)	32·7	15·6	85	118	1·6

was found to be $9{\cdot}46 \times 10^{-3}$ min.$^{-1}$. The value of k' is thus given by

$$9{\cdot}46 \times 10^{-3} \times \frac{0{\cdot}65}{0{\cdot}37} \times \frac{2{\cdot}5}{3} \times \frac{0{\cdot}6}{0{\cdot}45} = 18{\cdot}4 \times 10^{-3}.$$

Similarly, a value of $15{\cdot}6 \times 10^{-3}$ for k' was found for the supernatant fraction, and so the percentage activity in this fraction is $15{\cdot}6/18{\cdot}4 = 85\,\%$.

Virtually all the γ-glutamyl lactamase activity is thus in the supernatant fraction of rabbit-liver cells. Moreover, as there are hydrolytic enzymes in the nuclear fraction (see below), it follows that γ-glutamylglycine is not hydrolysed at all rapidly by these enzymes.

Synthesis of ophthalmic acid

An enzyme preparation from rabbit liver, as used by Snoke & Bloch (1955) for the synthesis of glutathione, catalysed the synthesis of ophthalmic acid from the constituent amino acids. The product was identified by preparative paper electrophoresis; hydrolysis gave the three amino acids in equimolar proportions. The course of the synthesis is illustrated in Fig. 2. Now the synthesis of ophthalmic acid in extracts from calf lens proceeds by the steps (Cliffe & Waley, 1958*a*):

$$\text{Glu} + \text{AB} + \text{ATP} \rightarrow \gamma\text{-Glu.AB} + \text{ADP} + \text{P}$$

$$\gamma\text{-Glu.AB} + \text{Gly} + \text{ATP} \rightarrow \text{Ophthalmic acid} + \text{ADP} + \text{P}$$

where AB stands for α-amino-*n*-butyric acid, ADP adenosine diphosphate, and P phosphate. The shape of the lower curve in Fig. 2 suggests that there are also two steps, and that they proceed at comparable rates.

In the experiments with extracts of calf lens, the presence of cysteine depressed the rate of synthesis of ophthalmic acid (Cliffe & Waley, 1958*a*), and the same effect has now been found with liver extracts (Fig. 3). When cysteine is present, the synthesis of glutathione is competing with the synthesis of ophthalmic acid. This suggests that one (or, possibly, both) of the enzymes that catalyse the synthesis of glutathione also plays a part in the synthesis of ophthalmic acid.

In the synthesis of glutathione, it is important that the ADP formed should be reconverted into ATP, and the same may be expected to be true for the synthesis of ophthalmic acid. A relatively high concentration of 3-phosphoglyceric acid was therefore used; excess of 3-phosphoglyceric acid, however, depressed the extent of synthesis of ophthalmic acid, and the optimum concentration was about 33 mM. Variation of the concentration of

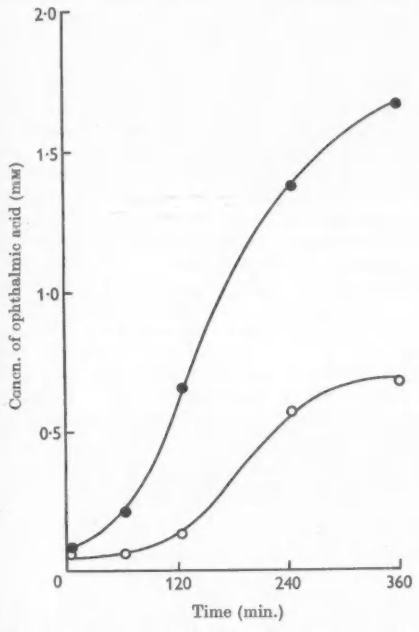

Fig. 2. Formation of ophthalmic acid from its constituent amino acids, catalysed by an extract of acetone-dried rabbit liver, in the presence (●) and absence (○) of dilute, dialysed, lens extract.

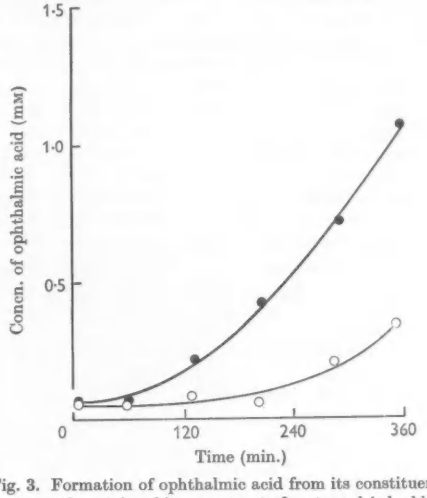

Fig. 3. Formation of ophthalmic acid from its constituent amino acids, catalysed by an extract of acetone-dried rabbit liver, in the presence (●) and absence (○) of cysteine.

ATP (in the range 1·5–8 mM) did not affect the rate of synthesis of ophthalmic acid. Lens extracts are active in the conversion of phosphoglyceric acid and ADP into ATP, and so we examined the effect of adding a dilute lens extract to the enzyme preparation, from rabbit liver, which was being used for the synthesis of ophthalmic acid. The synthesis of ophthalmic acid was, in fact, accelerated by the addition of the lens extract (Fig. 2, upper curve). The lens extract used was so dilute that, on its own, it did not catalyse the synthesis of detectable amounts of ophthalmic acid. The dilute lens extract was shown to convert 3-phosphoglyceric acid and ADP into ATP. The reaction was followed by ion-exchange chromatography (Cohn & Carter, 1950), and three-quarters of the added ADP was converted into ATP after 1 hr. at 37°. It is by no means certain that the effect of added lens extract on the synthesis of ophthalmic acid by the liver extract is due to the factors discussed above; in practice, however, the effect was useful.

A few experiments were carried out with extracts from the livers of other animals. Extracts of calf liver were considerably less active than those of rabbit liver; pigeon-liver extracts were comparable with rabbit-liver ones.

The intracellular distribution of the enzymes that catalyse the synthesis of ophthalmic acid (and of glutathione) have been studied. The results of the fractionation, from assays carried out with added (dilute) extracts of lens, were as follows. The activities in the nuclear fraction, the mitochondria and the microsomes were low and may be attributed to contamination by the supernatant fraction, which contains about 40 % of the total activity of the unfractionated tissue. The recovery is not high, but this is not surprising as the system is complex (some five enzymes must be present), and the manipulations prolonged. During the course of centrifuging, acetone precipitation and dialysis, there is some likelihood of loss in activity, which may be greater in one of the fractions than it is in the unfractionated tissue. The simplest conclusion is that the enzymes that catalyse the synthesis of ophthalmic acid are present only in the supernatant fraction. Similarly, the only subcellular fraction in which the synthesis of glutathione could be demonstrated was the supernatant.

Breakdown of glutathione and of ophthalmic acid

Some conclusions reached later are anticipated here: there are two enzymes that catalyse the breakdown of ophthalmic acid; these are γ-glutamyl lactamase and γ-glutamyl transpeptidase. The results of the subcellular fractionation of the enzymes that catalyse the breakdown of the tripeptides is shown in Table 2. Two points are immediately apparent. The first is that the breakdown of glutathione occurs mainly in the nuclear fraction. The second is that the breakdown of ophthalmic acid occurs both in the nuclear fraction and the supernatant. The products of the breakdown of ophthalmic acid in the nuclear fraction and in the supernatant differ: glutamic acid is a product in the nuclear fraction, and 5-oxypyrrolidine-2-carboxylic acid in the supernatant fraction. The latter compound is characteristic of the action of γ-glutamyl lactamase, and this, as we have seen, is localized in the supernatant fraction. Hence the breakdown of ophthalmic acid in the supernatant fraction is ascribed to the action of γ-glutamyl lactamase. This conclusion was confirmed by the observation that the presence of γ-glutamylglycine depressed the rate of breakdown of ophthalmic acid in the supernatant fraction. Glutathione, since it is not broken down at an appreciable rate in the supernatant fraction, must be more resistant to γ-glutamyl lactamase.

We are now in a position to compare the distribution of the enzyme (γ-glutamyl transpeptidase) that catalyses the hydrolysis of glutathione and of ophthalmic acid. From Table 2, the γ-glutamyl lactamase in the supernatant accounts for about half the activity in the breakdown of opththalmic acid. The distribution of the other (hydrolytic) enzyme may therefore be obtained by doubling the values for the activities of the nuclear fraction, mitochondria and microsomes given in the second column of Table 2. Comparison with the values for the breakdown of glutathione shows that the

Table 2. *Distribution of enzymes that catalyse the breakdown of glutathione and of ophthalmic acid in subcellular fractions of rabbit liver*

The activity of each fraction is expressed as a percentage of the activity of the unfractionated extract.

	Breakdown of		Calculated hydrolysis of ophthalmic acid
Fraction	Glutathione	Ophthalmic acid	
Nuclei + unbroken cells	75	37	74
Mitochondria	12	4	8
Microsomes	14	9	18
Supernatant	0	47	—
Total	101	97	—

distribution of the two activities is now very similar. This suggests that the same enzyme may catalyse the breakdown of both tripeptides. This conclusion was confirmed by the observation that the presence of glutathione depressed the rate of breakdown of ophthalmic acid in the nuclear fraction.

Transpeptidations catalysed by the nuclear fraction

On incubation of ophthalmic acid and glycylglycine with rabbit-liver extracts, a new ninhydrin-positive, acidic, species was formed (Fig. 4). This product is probably γ-glutamylglycylglycine (γ-Glu.Gly.Gly), formed by transpeptidation:

Transpeptidation takes place more rapidly when the nuclear fraction from rabbit liver is used (Fig. 5). The acidic product was isolated by preparative paper electrophoresis; it was chromatographically homogeneous, and after acid hydrolysis the molar ratio of the amino acids (estimated by quantitative paper chromatography) was glutamic acid 1·0, glycine 1·9. This is consistent with our formulation of the product as γ-glutamylglycylglycine.

The rate of breakdown of ophthalmic acid (in the nuclear fraction) was about 30 times as great in the presence of glycylglycine (20 mM) as in its absence.

$$\gamma\text{Glu.AB.Gly} + \text{Gly.Gly} \rightarrow \gamma\text{-Glu.Gly.Gly} + \text{AB.Gly}$$

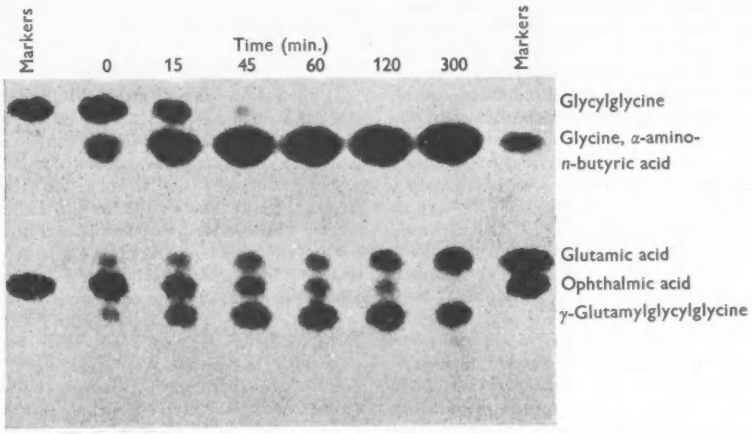

Fig. 4. Electrophoresis paper showing the course of the reaction between ophthalmic acid and glycylglycine, catalysed by an extract of rabbit liver. The overall reaction is

$$\gamma\text{-Glu.AB.Gly} + \text{Gly.Gly} \rightarrow \gamma\text{-Glu.Gly.Gly} + \text{AB} + \text{Gly}.$$

Electrophoresis was carried out at pH 4, and travel towards the anode is downwards in the illustration.

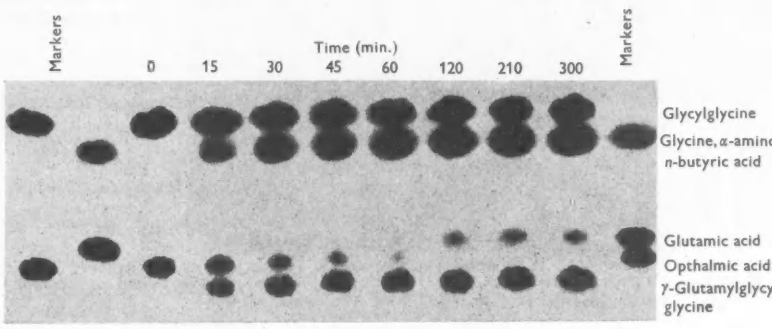

Fig. 5. Electrophoresis paper showing the course of the reaction between ophthalmic acid and glycylglycine, catalysed by the nuclear fraction from rabbit liver. The reaction and procedure were as in Fig. 4.

Thus with a relatively high concentration of an 'acceptor', transpeptidation becomes the main route of breakdown. On comparison of Figs. 4 and 5, the glycylglycine may be seen to be stable in the nuclear fraction, but broken down quite rapidly in the extract from whole liver. In rat liver, the enzyme that catalyses the hydrolysis of glycylglycine is in the supernatant fraction (Rademaker & Soons, 1957), and the same may well be true for rabbit liver. Hence transpeptidation proceeds especially rapidly in the nuclear fraction, both because the enzyme (γ-glutamyl transpeptidase) is present in this fraction, and because the glycylglycine is stable in this fraction. The instability of the 'acceptor' for transpeptidation in whole liver precludes any reliable measurement of the intracellular distribution of the enzyme that catalyses the transpeptidation.

The transpeptidation that has just been described leads to the breakdown of ophthalmic acid. Another transpeptidation that has been carried out leads to the formation of ophthalmic acid. When glutathione and α-amino-*n*-butyrylglycine (AB.Gly) are incubated with the nuclear fraction, ophthalmic acid is formed:

$$\gamma\text{-Glu.CySH.Gly} + \text{AB.Gly} \quad \text{(glutathione)}$$
$$\downarrow$$
$$\gamma\text{-Glu.AB.Gly} + \text{CySH.Gly} \quad \text{(ophthalmic acid)}$$

The course of this reaction is shown in Fig. 6. Here the samples from the reaction mixture were oxidized by performic acid to convert glutathione (GSH) into the sulphonic acid (GSO_3H), which has a relatively high mobility (towards the anode) on electrophoresis at pH 4, whereas GSH and ophthalmic acid have nearly the same mobility. Ophthalmic acid is seen to be formed rapidly; it is also broken down, so that its concentration does not change greatly after the reaction has proceeded for several minutes.

The substance with the highest mobility towards the anode in Fig. 6 is cysteic acid, which is formed from cysteine (or cystine) in the reaction mixture by the oxidation with performic acid. Although cysteinylglycine (CySH.Gly) was written as a product in the transpeptidation between glutathione and α-amino-*n*-butyrylglycine, the cysteinylglycine seems to be hydrolysed to the amino acids, presumably by cysteinylglycine dipeptidase, which is commonly present in tissues.

Comparison of tissues

Table 3 shows some results obtained with tissues other than rabbit liver. The variation in the activity of the enzyme that catalyses the breakdown of glutathione is quite marked, and for the liver, the activity depends on the species (Neubeck & Smythe, 1944). The results for ophthalmic acid are similar to those for glutathione, and presumably it it generally true that the same enzyme (γ-glutamyl transpeptidase) attacks both the tripeptides. The dipeptide, γ-glutamylglycine, gives 5-oxopyrrolidine 2-carboxylic acid (and glycine) with rat-liver, or rabbit-lens, extracts; hence here the enzyme is γ-glutamyl lactamase. With sheep-kidney extracts, however, the products are glutamic acid and glycine, so that here the enzyme responsible for the breakdown is γ-glutamyl transpeptidase.

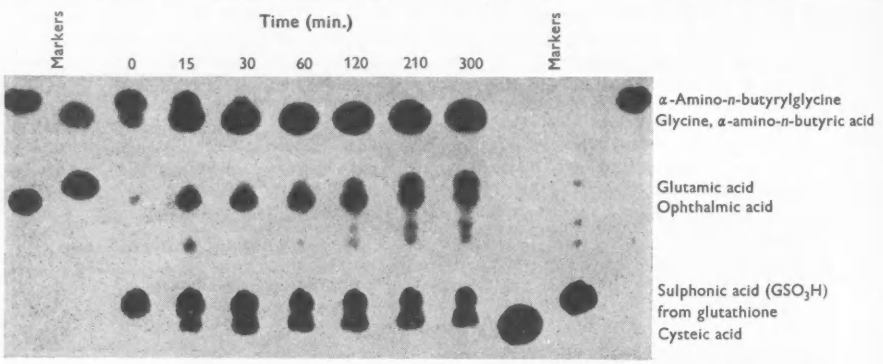

Fig. 6. Electrophoresis paper showing the course of the reaction between glutathione and α-amino-*n*-butyrylglycine, catalysed by the nuclear fraction from rabbit liver. The overall reaction is:

$$\gamma\text{-Glu.CySH.Gly} + \text{AB.Gly} \rightarrow \gamma\text{-Glu.AB.Gly} + \text{CySH} + \text{Gly}$$

The thiols (GSH and CySH) have been oxidized to the corresponding sulphonic acids (GSO_3H and $CySO_3H$) before electrophoresis. Procedure as in Fig. 4.

Table 3. *Breakdown of γ-glutamyl peptides in tissues*

The reaction mixtures (total volume, 0·6 ml.) were 5 mM with respect to glutathione, or ophthalmic acid, or 10 mM with respect to γ-glutamylglycine, and contained 0·45 ml. of extract of the tissue. The tissues were minced, and the mince was ground with phosphate buffer (see Methods) (2 ml. of buffer/g. of mince). The units of the rates are $10^3k'$ (see Table 1). The reaction mixtures were incubated at 37° and pH 7·4 for 5 hr.

Tissue	Glutathione	Ophthalmic acid	γ-Glutamylglycine
Guinea-pig liver	109	—	—
Rabbit liver	10–30	10–33	29–49*
Rat liver	<1	<1	Active*
Sheep kidney	>200	>200	80†
Rabbit lens	<1	<1	8*

* The product is 5-oxopyrrolidine-2-carboxylic acid. † The product is glutamic acid.

Occurrence of ophthalmic acid in rat liver

Rat liver (35 g.) was macerated with cooled 8% (w/v) trichloroacetic acid (120 ml.) and the protein removed by centrifuging. The supernatant solution was freed from trichloroacetic acid by extraction with *N*-methyldioctylamine (20 ml.) in $CHCl_3$ (400 ml.), and excess of base removed by two further extractions with $CHCl_3$. The solution was concentrated to a volume of about 17 ml., and the pH raised to 7·6 with N-NaOH, before fractionation on a column (1·2 cm. × 30 cm. long) of Dowex 1 (acetate). The resin had been treated as previously described (Waley, 1959), converted into the acetate form, and washed with water. After the extract had been added to the column, the column was washed with water (50 ml.), and then eluted with pyridine–formate, pH 5·5 (4·2 ml. of 90% formic acid and 16·2 ml. of pyridine to 1 l.), at a rate of 12 ml./hr.; fractions of 3 ml. were collected. Fractions 35–47 were ninhydrin-positive, and were united and evaporated. Examination by paper chromatography and paper electrophoresis showed that, besides glutamic acid and aspartic acid, there was a spot which had the same mobility as that of ophthalmic acid. After hydrolysis in 6N-HCl at 110° for 16 hr., this spot was absent, but glycine and α-amino-*n*-butyric acid were now present. These results suggest the presence of ophthalmic acid in rat liver; the concentration, gauged by the intensity of the spot, is about 1 μmole/100 g. of liver.

DISCUSSION

γ-Glutamyl lactamase

The application of the technique of subcellular fractionation to the enzymes that catalyse the synthesis and breakdown of γ-glutamyl peptides has helped us to unravel these reactions, and also suggests a well-defined intracellular distribution of these enzymes. Centrifugal fractionation was used by Connell & Hanes (1956) in their purification of γ-glutamyl lactamase. They found that lactamase from pig liver was in the supernatant fraction, whereas the γ-glutamyl transpeptidase was in the precipitated ('particulate') fraction. Our results on the enzymes from rabbit liver confirm, and extend, those of Connell & Hanes (1956). The results of the quantitative study of the distribution of γ-glutamyl lactamase (Table 1) show that, within the limits of the procedure, the enzyme is solely present in the supernatant fraction. One result of this distribution is that the enzyme may affect the course of the synthesis of the γ-glutamyl tripeptides *in vivo* (see Cliffe & Waley, 1958*a*).

The range of activity of γ-glutamyl lactamase has not been widely investigated. Connell & Hanes (1956) record that γ-glutamylglycine, γ-glutamylglutamic acid, γ-glutamylphenylalanine and glutathione are all attacked, and that γ-glutamylglycine is attacked more rapidly than glutathione. We also find that γ-glutamylglycine is readily cleaved by the enzyme from rabbit liver, and that ophthalmic acid is broken down, too; glutathione is considerably more resistant. Calf lens contains a γ-glutamyl lactamase (Cliffe & Waley, 1958*a*), but neither here, nor with rabbit lens, is ophthalmic acid attacked at a detectable rate. Thus it seems to be generally true that γ-glutamyl lactamases attack γ-glutamylglycine more rapidly than they attack the tripeptide, ophthalmic acid, but this difference is much more marked with the enzyme from the lens than with the enzyme from liver.

Synthesis of the tripeptides

The enzyme system that catalyses the synthesis of ophthalmic acid, and of glutathione, is also confined to the supernatant fraction of rabbit liver. There are some indications (Hollemann, 1954) that the same is true for rat liver. This enzyme system is quite complex. There are the two stages in the synthesis of the tripeptide, and three stages in glycolysis (catalysed by phosphoglyceric acid mutase, enolase and pyruvic kinase) by which ATP is 'regenerated' from ADP. The second stage in the synthesis of glutathione (in extracts of rat liver) has recently been shown to involve 'soluble'

ribonucleic acid (RNA) (i.e. the RNA from the supernatant fraction) (Bates & Lipmann, 1960); the same mechanism presumably obtains in extracts of rabbit liver. From the results of our 'competition' experiments it seems quite likely that soluble RNA also plays a part in the synthesis of ophthalmic acid. If, in fact, γ-glutamyl-α-amino-*n*-butyric acid can be bound to the RNA at the same site as γ-glutamylcysteine normally is, then this 'latitude' in the synthesis of tripeptides forms a contrast with the specificity in the synthesis of proteins. Loftfield, Grover & Stephenson (1953) have shown that labelled α-amino-*n*-butyric acid, on incubation with rat-liver slices, is not incorporated into the proteins at a significant rate, although it enters the cells. Moreover, α-amino-*n*-butyric acid has not been detected in the hydrolysates of proteins. Since we know that extracts of several mammalian livers can catalyse the synthesis of ophthalmic acid, and that rat liver probably contains it, the system that brings about the synthesis of the tripeptide thus seems less selective than that which is responsible for protein synthesis.

Breakdown of glutathione in the nucleus

The enzyme (γ-glutamyl transpeptidase) that catalyses the hydrolysis of glutathione, and of ophthalmic acid, is concentrated in the nuclear fraction (Table 2); the specific activity (i.e. the activity per unit weight of protein) of this fraction is about three times that of the whole tissue. We now have to consider whether, in fact, the enzyme is localized in the nuclei of the cells of rabbit liver. The nuclear fraction is contaminated with whole cells, erythrocytes, a few mitochondria and some debris. The high specific activity of the nuclear fraction, together with the fact that over 90% of the cells were broken, makes it unlikely that all the activity in the nuclear fraction could be due to the few unbroken cells present. The activity cannot be due to erythrocytes, as the nuclear fraction from perfused liver was active, nor can it be due to mitochondria, as the specific activity of the mitochondrial fraction is less than that of the nuclear fraction. An enzyme might be adsorbed on a cell particle (rather than localized within it); this is how Rosenthal, Gottlieb, Gorry & Vars (1956) interpret their observation that arginase is no longer found in the nuclear and microsomal fractions when the sucrose medium contains metal ions. In our case, however, added metal ions did not lessen the activity of the nuclear fraction. A more homogeneous nuclear fraction was prepared by the procedure of Hogeboom *et al.* (1952), but this preparation did not catalyse the hydrolysis of glutathione. This procedure involves, at one stage, sieving the extract through lint. We found that this sieving removed at least half the nuclei, and also left little activity in the homogenate, even before differential centrifuging. We therefore regard the negative results obtained by this procedure as inconclusive. The simplest explanation of the high activity of the nuclear fraction does seem to be that the enzyme, γ-glutamyl transpeptidase, is localized within the nucleus of the cell. If so, this is an unusual occurrence (Roodyn, 1959). The enzyme that catalyses the synthesis of diphosphopyridine nucleotide (DPN) is localized in the nucleus (Hogeboom & Schneider, 1952) and Morton (1958) has suggested that the synthesis of DPN in the nucleus, and its breakdown in the cytoplasm, may be a factor that determines cell-division. Glutathione, on the other hand, may be synthesized in the cytoplasm and broken down in the nucleus. This conclusion must be regarded as tentative, as the interpretation of the results of fractionation by differential centrifuging is fraught with pitfalls (Allfrey, 1959). There is also the possibility that, *in vivo*, glutathione might be mainly broken down in the cytoplasm by γ-glutamyl lactamase; this process could be more rapid than the diffusion of glutathione into the nucleus, and hydrolysis within it.

Although there are no other reports of a quantitative study of the subcellular fractionation of the enzyme that catalyses the hydrolysis of glutathione, Lang, Siebert & Müller (1951) noted the presence of this enzyme in the nuclear fraction from pig kidney, and Hird & Springell (1954*a*), in their purification of the enzyme, used a 'particulate' fraction. Bailie & Morton (1958) detected γ-glutamyl transpeptidase in the microsomes from the mammary gland, and Binkley, Davenport & Eastall (1959) also found the enzymes to be mainly present in the microsomes from kidney; in plant tissues, the distribution seems somewhat different (Webster, 1953).

Transpeptidation

In extracts of animal tissues, γ-glutamyl transpeptidase catalyses the hydrolysis of glutathione, and also catalyses transpeptidation reactions (Hird & Springell, 1954*b*). Indeed, there is some evidence that transpeptidation occurs even without an added amino acid either by the reaction of (hydrolytically liberated) cysteine with glutathione (Hird & Springell, 1954*b*):

γ-Glu.CySH.Gly + CySH → γ-Glu.CySH + CySH.Gly

or by the reaction between two molecules of glutathione (Revel & Ball, 1959):

γ-Glu.CySH.Gly + γ-Glu.CySH.Gly → γ-Glu.γ-Glu.CySH.Gly + CySH.Gly

Our experiments show that both transpeptidation and hydrolysis are catalysed by the nuclear fraction and thus support the idea that γ-glutamyl transpeptidase catalyses both hydrolysis and transpeptidation. There is a considerable advantage in the use of the nuclear fraction to study transpeptidation, as the best 'acceptors' are dipeptides, such as glycylglycine (Fodor, Miller & Waelsch, 1953), and the dipeptides are more stable in the nuclear fraction (compare Fig. 4 with Fig. 5), as the dipeptidases are mainly in the supernatant fraction. The absence of γ-glutamyl lactamase from the nuclear fraction is also an advantage in the study of transpeptidation.

There is considerable variation in the γ-glutamyl transpeptidation activity of different organs. Hanes, Hird & Isherwood (1952) demonstrated transpeptidation with extracts from sheep kidney and pancreas, and Fodor *et al.* also used sheep brain and calf liver. Amongst a variety of tissues, Revel & Ball (1959) detected γ-glutamyl transpeptidase only in kidney and pancreas. Our results (Table 2) also show that sheep kidney was the most active of the tissues tested, although we were able to carry out transpeptidation quite readily with the nuclear fraction from rabbit liver. One such reaction (Fig. 6) led to the formation of ophthalmic acid from glutathione and α-amino-*n*-butyrylglycine:

$$\gamma\text{-Glu.CySH.Gly} + \text{AB.Gly} \rightarrow \gamma\text{-Glu.AB.Gly} + \text{CySH.Gly}$$

This reaction does not seem likely to contribute towards the synthesis of ophthalmic acid *in vivo*.

Composition and metabolism of the lens

The amounts of protein in the subcellular fractions of calf lens have been determined; in Table 4 these amounts are compared with the corresponding amounts from rabbit liver. There is a striking difference between the 'composition' of rabbit liver and calf lens: in the calf lens nearly all (96%) of the protein is found in the supernatant fraction. Indeed, the lens appears unique in the tissues so far studied, in the high proportion of protein in the supernatant fraction. Electron microscopy of lens fibres (Wanko & Gavin, 1959) shows the presence of nuclei, mitochondria and microsomes, but these become rather sparse in the fibres nearer the centre of the lens. The results of the differential centrifuging may be affected by proteins being extracted from the particles, but, whether or not this happens, a comparison of the results of subcellular fractionation of the lens and other tissues suggests that the lens would be expected to contain those enzymes that are commonly found in the supernatant fraction. On the other hand, those enzymes which are normally found in the nuclear, mitochondrial or microsomal fractions might be difficult to detect in lens extracts. This generalization does seem to hold quite well. Many of the enzymes that are concerned with glycolysis, for example, are quite active in the lens, and these enzymes are normally found in the supernatant fraction. Conversely, several of the enzymes of the tricarboxylic acid cycle are found in the mitochondrial fraction, and are barely detectable in lens extracts. Moreover, to revert to the metabolism of γ-glutamyl peptides, the enzymes that are present in the supernatant fraction from rabbit liver (γ-glutamyl lactamase, and the pair of enzymes responsible for the synthesis of glutathione, and of ophthalmic acid) are present in the lens, but γ-glutamyl transpeptidase cannot be detected in lens extracts.

Table 4. *Distribution of protein in the subcellular fractions of rabbit liver and of calf lens*

The amount of protein in each fraction is expressed as a percentage of the amount in the unfractionated extract. Protein was estimated by the method of Robinson & Hogden (1940).

Fraction	Rabbit liver	Calf lens
Nuclei + unbroken cells	21	0
Mitochondria	11	1
Microsomes	13	1
Supernatant	52	96
Total	97	98

SUMMARY

1. The intracellular distributions of the enzymes that catalyse the synthesis and breakdown of glutathione and its analogue, ophthalmic acid, have been studied. The tissue chosen was rabbit liver.

2. The synthesis of both tripeptides follows the same course, and competition experiments suggest that the same enzymes may be involved. These enzymes are present in the supernatant fraction of rabbit liver; no appreciable activity was detected in the other fractions.

3. The hydrolysis of glutathione is catalysed by the nuclear fraction; the other fractions have little or no activity. The nuclear fraction also catalyses transpeptidation reactions. Hydrolysis and transpeptidation are probably brought about by the same enzyme, γ-glutamyl transpeptidase. The problem of whether γ-glutamyl transpeptidase is localized in the nucleus is discussed.

4. Ophthalmic acid is broken down in both the nuclear and supernatant fractions. In the nuclear fraction, ophthalmic acid is hydrolysed to the con-

stituent amino acids (glutamic acid, α-amino-*n*-butyric acid, and glycine); transpeptidation also occurred in the nuclear fraction, and γ-glutamyl transpeptidase is the enzyme which brings about hydrolysis and transpeptidation in the nuclear fraction. The breakdown of ophthalmic acid in the supernatant fraction gives 5-oxopyrrolidine-2-carboxylic acid rather than glutamic acid, and is ascribed to the action of γ-glutamyl lactamase. The localization of γ-glutamyl lactamase in the supernatant fraction was shown when γ-glutamylglycine was used as substrate.

5. In the subcellular fractionation of calf lens, nearly all the protein is in the supernatant fraction. The enzymes that are most readily detected in lens extracts are those which are present in the supernatant fraction of liver.

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Studies on the Flash Photolysis of Visual Pigments

1. PIGMENTS PRESENT IN FROG-RHODOPSIN SOLUTIONS AFTER FLASH-IRRADIATION

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Irradiation of visual-pigment solutions with brief intense bursts of light makes it possible to detect products of bleaching which have only a transient existence or which are themselves photoreactive. Ideally, the requirement is for an instantaneous flash of intensity sufficient to excite all the visual-pigment molecules: in practice, electronically-operated discharge tubes of the type used in photography provide the best available light sources. Such tubes are capable of producing

flashes of high luminous intensity with durations ranging from a few microseconds to several milliseconds.

With a 64 J argon-filled flash-tube assembly delivering a 2–6 μsec. flash, Wulff, Adams, Linschitz & Abrahamson (1958) irradiated solutions of cattle rhodopsin. Specially designed equipment, which measured absorption spectra more or less immediately after the flash, revealed the presence of rapidly fading photoproducts with maxima displaced 12 mμ towards the blue. After about 1 hr. all thermal changes had ceased, and the solutions were found to contain a stable photosensitive substance with $\lambda_{max.}$ at 490 mμ: 8 mμ below that of the parent rhodopsin.

Solutions of frog rhodopsin were used in the present work and were exposed to the flash from a 400 J xenon-filled discharge tube. These flashes were of higher intensity but longer duration than those used by Wulff and his colleagues, yet the results are essentially similar. Initially produced thermally unstable pigments faded rapidly, and the material which remained after thermal changes were complete was stable and photosensitive. When this stable material was tested for homogeneity by the method of partial bleaching (Dartnall, 1952) two photosensitive pigments were detected.

METHODS

Preparation of frog-rhodopsin solutions

Frogs (*Rana temporaria*) were dark-adapted for about 16 hr. and killed by decapitation, and the retinae removed in dim red light (Lythgoe, 1937). The retinae were then washed three times with McIlvaine's buffer, pH 4·6, and extracted twice with aqueous 2% (w/v) digitonin solution (powdered digitonin obtained from L. Light and Co. Ltd.). Sufficient saturated sodium borate solution was added to the resultant extract to bring the pH to 8·2–8·7. Extracts prepared in this manner had $E_{min.}/E_{max.}$ ranging from 0·30 to 0·59.

One extract was prepared by floating off the rod outer segments in isopycnotic sucrose solution (Saito, 1938) and then proceeding as for whole retinae.

Hydroxylamine solutions

Aqueous hydroxylamine (0·1 M) was prepared from the hydrochloride and neutralized (to pH 7·0) by the addition of a few drops of concentrated sodium hydroxide solution.

Absorption spectra

In the experiments designed to measure thermally unstable pigments a Beckman DK-2 automatically recording spectrophotometer was used (1 cm. silica cells). With this apparatus spectra from 620 to 380 mμ were measured in about 1–1½ min. Thermally stable material was examined in a Unicam SP. 500 spectrophotometer (1 cm. glass cells) or, when partial-bleaching investigations were carried out, in a spectrophotometer (0·5 cm. glass cells) similar in principle to that described by Dartnall (1952). With the DK-2 and SP. 500 measurements were made at 25±3°. With the author's spectrophotometer the temperature was maintained at 25±0·5°.

Bleaching apparatus

Flash equipment. The flash tube was a xenon-filled Siemens SF E.22, operated from a Majorette power pack supplied for this purpose by Clive Courtenay and Co. Ltd. The energy dissipated through the tube in all experiments was 400 J. Light-output was found to be reproducible within 1%, and the duration of the flash was about 4 msec. (see Fig. 1).

The tube was in the form of a tight helix of 1¾ turns and about 6 cm. diam. It was surrounded by a ground-glass cylinder surmounted by a disk carrying an optical-cell holder. During a flash the silica or glass cell filled with rhodopsin solution was suspended in the centre of the helix. Thus light from the discharge tube irradiated the solution through both optical faces. The temperature was 25±3°. Although of such high intensity, the flash produced no detectable change in the temperature of the solution.

Partial-bleaching equipment. The author's spectrophotometer was designed as a dual-purpose instrument. By widening the monochromator entry and exit slits, changing to an intense ribbon-filament light-source and using the appropriate stray-light filters, solutions could be exposed to monochromatic radiation. A mirror was placed behind the glass optical cells during all bleaching operations, thus effectively doubling the light-intensity. The temperature was maintained at 25±0·5°.

Normal white bleaching. Normal white bleaching was used to convert all photosensitive pigments into indicator yellow. This was carried out by exposing solutions for 15 min. to the radiation from a 15 w lamp at a distance of about 1 ft. (illumination ~ 25 ft.-candles).

RESULTS

In Fig. 2 are shown the changes in absorption spectrum after flash-irradiation of frog rhodopsin. Curve *A* is the pre-flash spectrum. Measurement of curve *B* was commenced within 30 sec. of exposure to the flash and took about 1 min. to complete. The $\lambda_{max.}$ of this curve is at 478 mμ. Allowing 8 mμ correction for $E_{min.}/E_{max.}$ (Collins & Morton, 1950; Crescitelli & Dartnall, 1954), the

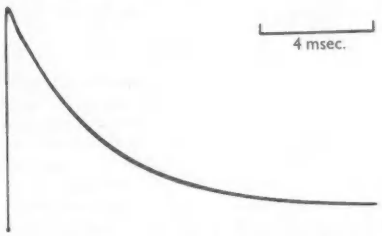


Fig. 1. Light-output curve of xenon-filled discharge tube.

true $\lambda_{max.}$ is estimated to be at 486 mμ. Curve *C* ($\lambda_{max.}$ after 10 mμ $E_{min.}/E_{max.}$ correction = 493 mμ) was obtained after 1½ hr. in darkness. A considerable loss in extinction had occurred at wavelengths longer than about 405 mμ, the difference spectrum for this process having $\lambda_{max.}$ at approx. 477 mμ. Subsequently, the extinction of the solution remained constant, but what remained was photosensitive, as is shown by the further fall in extinction to curve *D* after exposure to ordinary white light. The difference spectrum of this material showed a maximum at about 494 mμ.

The difference spectra of the fading process (henceforward called Fraction 1), the residual thermally stable photosensitive material (henceforward called Fraction 2) and the parent frog rhodopsin are compared in the lower half of Fig. 2. The rhodopsin difference spectrum has been obtained by subtracting the final fully bleached curve *D* from the pre-flash curve *A*; it is, of course, independent of any intervening processes.

In another similar experiment, the light of the flash was passed through an orange Ilford 201 filter before irradiating the solution. Fraction 1 was spectroscopically identical with that produced by an unfiltered flash. Fraction 2, however, although produced in substantially the same quantity as by the unfiltered flash, had $\lambda_{max.}$ as high as 500 mμ. This observation is considered further in the next paper (Bridges, 1961).

Effect of hydroxylamine. The quality and quantity of Fraction 2 are not altered by the presence of 0·01 M- or 0·03 M-hydroxylamine. Thus in experiments on solutions without hydroxylamine Fraction 2 had $\Delta E_{max.}$ 32·45 ± 2·07 % of that of the parent rhodopsin; in the presence of hydroxylamine this percentage was 32·22 ± 0·99. Again, the difference spectra of Fraction 2 produced in the presence and the absence of hydroxylamine were identical from 620 to about 480 mμ. Divergence of the curves below this point (Fig. 3) was attributed to the differing absorptions of the photoproducts retinene oxime and alkaline indicator yellow (*N*-retinylidene opsin).

Is Fraction 2 a mixture of photosensitive pigments?

Fig. 4 illustrates a typical experiment designed to test the homogeneity of Fraction 2. Curve *B* is the absorption spectrum of a solution of frog rhodopsin measured 1½ hr. after a flash. All thermal changes were completed, as shown by the fact that the return measurements from 610 to 390 mμ follow the curve of the outward measurements from 380 to 620 mμ (cf. Dartnall, 1952). Spectra *C*, *D* and *F* are respectively after irradiation for 1, 5 and 3 hr. with λ600 mμ light (irradiance of the order of 10^{13} quanta sec.$^{-1}$ cm.$^{-2}$). The spectrum *E* was measured after the solution had been left

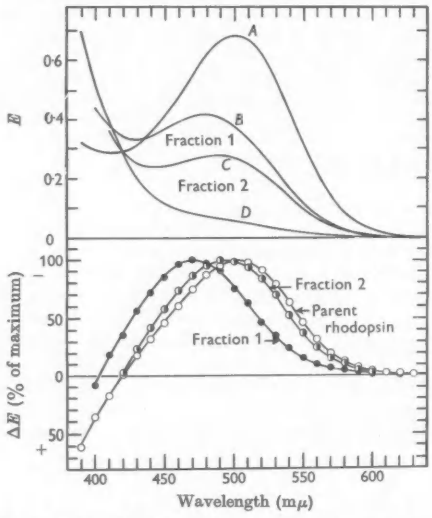


Fig. 2. Effect of flash-irradiating a solution of frog rhodopsin. Curve *A*, initial absorption spectrum; curve *B*, absorption spectrum of initial photoproduct: this curve was measured from 600 to 400 mμ (the reading at 500 mμ was 30 sec. after the flash); curve *C*, after 1½ hr. in darkness; curve *D*, after exposure to normal white light (all spectra measured on automatic recorder). The lower half of the Figure shows the following difference spectra: parent pigment (*D* minus *A*); Fraction 1 (*C* minus *B*); Fraction 2 (*D* minus *C*).

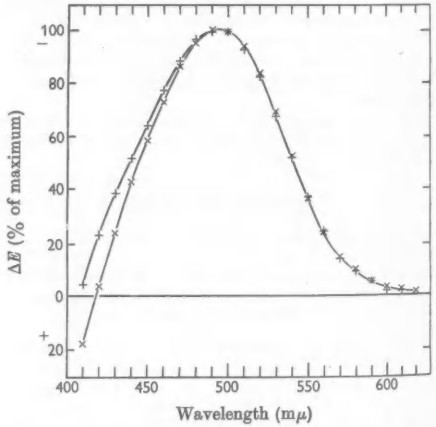


Fig. 3. A comparison of difference spectra produced when Fraction 2 is bleached with white light: +, in the presence of 0·01 M-hydroxylamine; ×, no hydroxylamine added.

overnight in darkness and was due to an alteration in irrelevant absorption. Since this experiment was carried out in the presence of 0·01 M-hydroxylamine, no regeneration could occur. Finally, curve *G* is the absorption spectrum of the solution after exposure to a 15 W lamp for 15 min.

In the lower half of Fig. 4 are shown the difference spectra of each bleaching operation, namely $C-B$, $D-C$, $F-E$ and $G-F$. Their maxima shift from 502 mμ for the first λ600 mμ irradiation to 486 mμ for the final white bleach. This experiment, and the others listed in Table 1, clearly show that two photosensitive pigments are present in Fraction 2. The three difference spectra shown in Fig. 5 have been obtained by subtracting from the fully bleached absorption spectrum of a solution the corresponding absorption spectrum measured after exhaustive irradiation with long-wavelength light (curve $G-F$ of Fig. 4 is such a difference spectrum). These difference spectra are identical within experimental error and show that one of the photosensitive pigments has $\lambda_{max.}$ at 486 ± 2 mμ. Since this pigment cannot be extracted from frog retina, and therefore has no claim to be a visual pigment, it is proposed to call it photosensitive pigment 486 or P 486. In this way it can be distinguished from a truly visual pigment, e.g. frog rhodopsin or VP 502.

The other pigment of Fraction 2 seems to be identical with the parent frog rhodopsin, for, as Fig. 6 shows, the difference spectrum resulting from an initial short red irradiation and that produced when frog rhodopsin is bleached are identical. The difference spectrum produced when Fraction 2 is bleached by exposure to white light (as in Fig. 2) is the sum of the difference spectrum produced by bleaching the component pigments. In Fig. 7 the difference spectra produced by bleaching pure P 486 and frog rhodopsin respectively are shown scaled according to the proportions in which they occur in Fraction 2. The figure represents the mean of nine results, in which it was found that P 486 accounted for $52{\cdot}3 \pm 3{\cdot}2$ % of the total photosensitive material. In terms of the $\Delta E_{max.}$ values, the total photosensitive pigment in Fraction 2 amounted to $32{\cdot}87 \pm 1{\cdot}59$ % of the pre-flash rhodopsin. (It should be noted that the overall $\Delta E_{max.}$ for Fraction 2 amounts to only 98·4 % of the summed $\Delta E_{max.}$ values of the component difference spectra.)

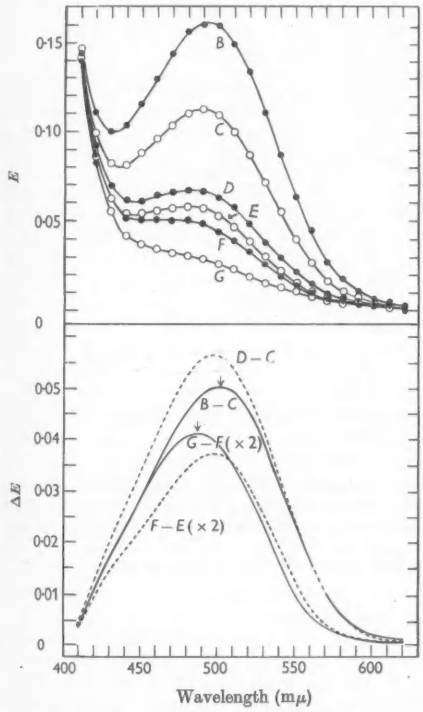


Fig. 4. The non-homogeneity of Fraction 2. Upper half of Figure: curve *B*, absorption spectrum of a solution of frog rhodopsin 1½ hr. after a flash; curves *C*, *D* and *F*, after 1, 5 and 3 hr. irradiation respectively with λ600 mμ light; curve *G*, after irradiation with normal white light; curve *E*, after leaving the solution overnight in darkness. Lower half of Figure: the difference spectra for each bleaching operation (the solution contained 0·01 M-hydroxylamine).

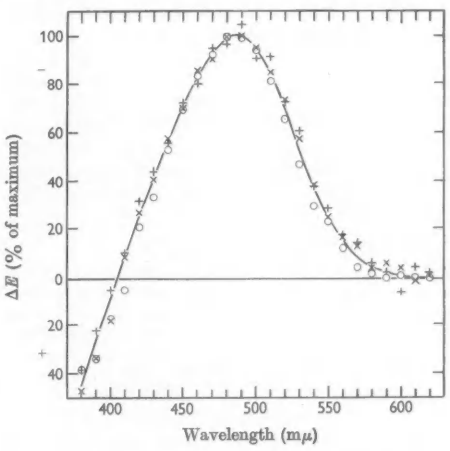


Fig. 5. Difference spectra of terminal white bleachings after the exhaustive irradiation of Fraction 2 with long-wavelength light. Corresponding experiments are listed in Table 1. ○, Expt. no. 3; +, expt. no. 4; ×, expt. no. 5.

Table 1. *Summary of experiments showing the presence of two photosensitive pigments in Fraction 2*

Stray light filters were used in conjunction with the monochromator. Ilford filters 203 and 201 were used for λ600 mμ and λ580 mμ bleaches respectively.

	Expt. no.	Wavelength of bleaching light (mμ)	Duration (hr.)	$\Delta E_{max.}$ (0·5 cm.)	$\lambda_{max.}$ (mμ)
No hydroxylamine	1	600	1	0·0295	500
		580	¾	0·0375	498
		White	½	0·0460	491
	3	600	4	0·0733	502
		600	2	About 0·0014	About 500
		580	1	0·0114	490
		White	¼	0·0292	485
Hydroxylamine (0·01 M)	4	600	2	0·0620	502
		580	1	0·0287	498
		580	2	0·0220	494
		White	¼	0·0194	488
	5	600	1	0·0497	502
		600	5	0·0560	497
		600	3	0·0185	495
		White	¼	0·0204	486

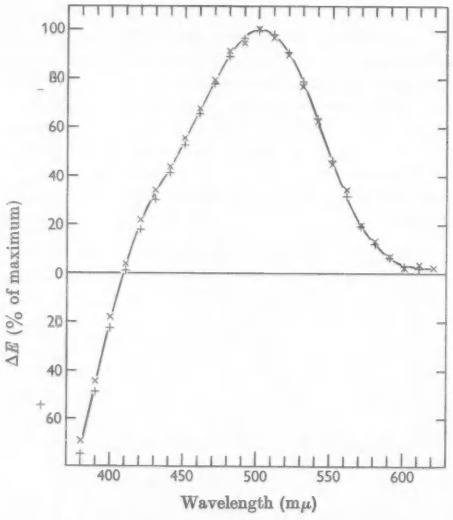


Fig. 6. Correspondence between the difference spectrum produced when frog rhodopsin is bleached in the presence of 0·01 M-hydroxylamine (+) and that produced by an initial red bleaching of Fraction 2 (×) i.e. curve *C* minus *B* of Fig. 4.

Effect of the flash on a freshly bleached solution of frog rhodopsin

A solution of frog rhodopsin was exposed to white light until all the visual pigment had been converted into alkaline indicator yellow. This material was then flash-irradiated, left in darkness for 1 hr. and its absorption spectrum measured.

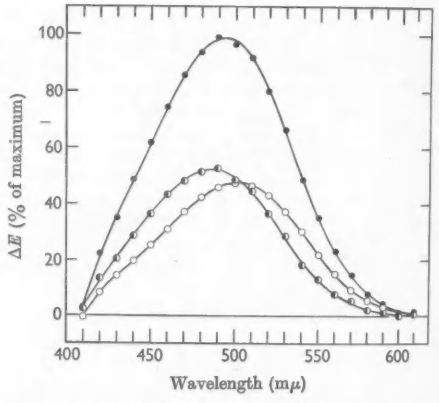


Fig. 7. Composition of Fraction 2. ●, Difference spectrum produced when Fraction 2 is bleached with white light; ◐, difference spectrum produced when pure pigment 486 is bleached; ○, difference spectrum produced when frog rhodopsin is bleached (0·01 M-hydroxylamine present).

The flash produced a fall in extinction at short wavelengths, but a very small rise in extinction beyond about 450 mμ. This rise was maximal at about 500 mμ and amounted to only about 3% of the original rhodopsin. Exposure of this solution to white light caused bleaching, showing that a photosensitive pigment had been generated by the flash. Its difference spectrum (obtained by subtracting the post-flash absorption spectrum from that of the final fully bleached solution) was identical with that of frog rhodopsin (Fig. 8).

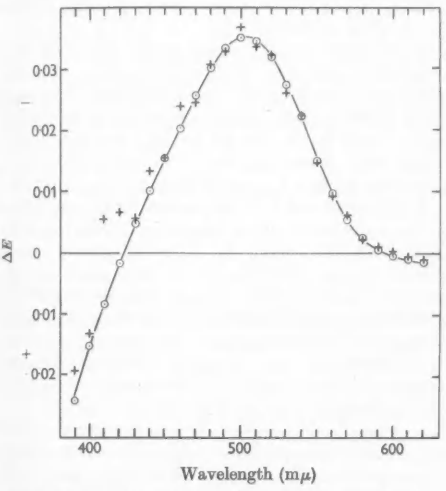


Fig. 8. Nature of the photosensitive pigment produced by flash-irradiation of indicator yellow. A solution of frog rhodopsin ($E_{500\ m\mu}$ 1·4685) was completely bleached by exposure to normal white light. The indicator yellow produced was then flash-irradiated and left for 1 hr. +, Extinction changes (differences pectrum) which took place on further exposure to white light; ⊙, difference spectrum of frog rhodopsin when bleached by a similar exposure to white light.

DISCUSSION

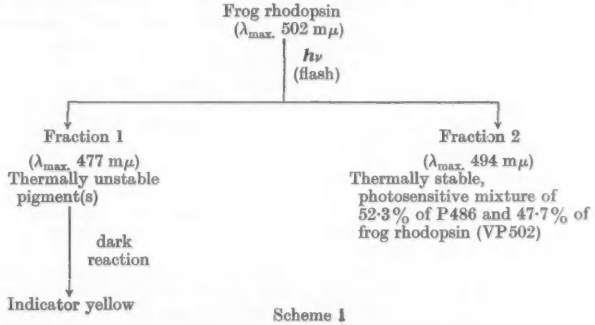
In the experiments described here, frog-rhodopsin solutions were exposed to intense flashes of light lasting about 4 msec. About ½ min. later, when measurements were begun, the extinction at all wavelengths above 405 mμ was found to be decreasing. After an hour in darkness no further changes were observed, and the solution contained roughly equal amounts of frog rhodopsin and a new photosensitive pigment with $\lambda_{max.}$ at 486 mμ.

Wulff and his colleagues suggested that the initial photoproduct of flash-irradiation of cattle rhodopsin consisted of two fractions. One of these (Fraction 1) decomposed in darkness at room temperature; the other (Fraction 2) was photosensitive and thermally stable. The present observations can be interpreted along similar lines, although another possibility should be considered. Low-temperature studies (Bridges, 1960) have shown that a photoproduct of frog rhodopsin (with $\lambda_{max.}$ at about 480 mμ at −5°) can be thermally converted into the parent photosensitive pigment. It could be therefore that part of Fraction 2 is generated during the thermal-decay process. In the low-temperature work conversions of this nature were characterized by a bodily shifting of the absorption spectrum towards longer wavelengths as the photoproduct changed to a stable photosensitive pigment. This effect would be scarcely noticeable, provided that only a small proportion of Fraction 1 was converted into a Fraction 2 with little change of $\lambda_{max.}$. However, careful scrutiny of the present results and also those of Wulff *et al.* has revealed no evidence to support this notion.

The situation seems more akin to the low-temperature studies of Wald, Durell & St George (1950) and of Kropf & Hubbard (1958), where it is likely that the product of low-temperature irradiation of cattle rhodopsin is a mixture of thermolabile and thermostable photosensitive pigments (the latter resembling Fraction 2).

In all probability therefore the constituents of Fraction 2 are present throughout the post-flash period, according to Scheme 1.

Unpublished observations by the author (from experiments similar to that of Fig. 2) show that other visual pigments react similarly to flash-irradiation. Rat rhodopsin ($\lambda_{max.}$ 498 mμ) yields a Fraction 1 with $\lambda_{max.}$ at about 473 mμ and Fraction 2 with $\lambda_{max.}$ at 490 mμ. The vitamin A_2 porphyropsin of perch (*Perca fluviatilis*; $\lambda_{max.}$ about 541 mμ) also yields an unstable Fraction 1 and a



Scheme 1

stable photosensitive Fraction 2: both have maxima at about 525 mμ.

Part of the frog rhodopsin in Fraction 2 may be unbleached parent pigment: the remainder, together with P486, is generated during the flash period or very shortly after it (within a few microseconds in Wulff's experiments). Regeneration from irradiated indicator yellow cannot account for Fraction 2 since its quality and quantity are unaffected by hydroxylamine, which binds the retinene of indicator yellow and renders it incapable of regeneration. In any case, flash-irradiation of indicator yellow yields only a very small quantity of rhodopsin. The mechanism by which Fraction 2 is produced is considered further in the next paper (Bridges, 1961).

Pigment 486

It was suggested by Wulff *et al.* (1958) that their Fraction 2 was a mixture of cattle isorhodopsin (based on isoretinene$_1a$) and cattle rhodopsin (based on neoretinene$_1b$). Cattle rhodopsin has $\lambda_{max.}$ at 498 mμ, cattle isorhodopsin at 487 mμ (Hubbard & Wald, 1952). Thus on changing the steric configuration of the retinene isomer in cattle rhodopsin one obtains a new pigment with $\lambda_{max.}$ shifted by 11 mμ towards the blue. The $\lambda_{max.}$ of P486 is 16 mμ below that of frog rhodopsin, and it is quite possible that it bears a similar relationship to frog rhodopsin as does cattle rhodopsin to isorhodopsin, i.e. the retinene isomer has been altered. Isorhodopsin is understood to refer to a synthetic photosensitive pigment based on isoretinene$_1a$. Since, however, the relevant retinene isomers have not yet been characterized for the frog pigments, it seems advisable to retain the term P486 for the flash-generated photosensitive derivative described here.

SUMMARY

1. Alkaline (pH 8·2–8·7) aqueous digitonin solutions of frog rhodopsin at 25° were exposed to intense 4 msec.-duration flashes from a 400 J xenon-filled discharge tube.

2. Two types of pigment were found in the irradiated solutions. One, with $\lambda_{max.}$ at 477 mμ, was thermally unstable (Fraction 1) and, when left in darkness at 25°, decayed to alkaline indicator yellow over the course of about 1 hr. The other, which was thermally stable and photosensitive (Fraction 2), had $\lambda_{max.}$ at 494 mμ.

3. When Fraction 2 was tested for homogeneity by the method of partial bleaching, it was found to consist of two pigments. One of these, which accounted for $52{\cdot}3 \pm 3{\cdot}2$% of the total photosensitive material, had $\lambda_{max.}$ at 486 ± 2 mμ (photosensitive pigment 486). The other was identical with the parent frog rhodopsin.

4. Fraction 2 was not altered in composition or amount when produced in solutions containing hydroxylamine (0·01 or 0·03 M).

5. Flash-irradiation of alkaline indicator yellow (i.e. an alkaline solution of frog rhodopsin bleached to completion with normal white light) generated only a very small quantity (about 3%) of the parent rhodopsin.

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Studies on the Flash Photolysis of Visual Pigments

2. PRODUCTION OF THERMALLY STABLE PHOTOSENSITIVE PIGMENTS IN FLASH-IRRADIATED SOLUTIONS OF FROG RHODOPSIN

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Wulff, Adams, Linschitz & Abrahamson (1958) and Bridges (1961) have described the production of various pigments by flash-irradiating solutions of cattle and frog rhodopsin. In each case it was found that, after exposure to a flash, the solutions contained two types of pigment. One type, which decayed rapidly in darkness at room temperature, was called Fraction 1. The other, which was thermally stable and photosensitive, was called Fraction 2. The Fraction 2 of frog rhodopsin was shown (Bridges, 1961) to be a mixture of frog rhodopsin and a new pigment, probably based on another retinene isomer, which had $\lambda_{max.}$ at 486 mμ.

The present work is concerned with the composition and yield of Fraction 2 produced by flashes of various colours and intensities, and in solutions at different temperatures. The results of these experiments suggest a possible sequence of reactions occurring during and after the period of irradiation.

METHODS

Preparation of frog-rhodopsin solutions. Aqueous digitonin extracts from the dark-adapted retinae of frogs (*Rana temporaria*) were prepared in the manner described in the preceding paper (Bridges, 1961). For work at temperatures below 0°, where such solutions would freeze, glycerol (50 or 65%, v/v) was added (cf. Broda & Goodeve, 1941), and the pH was adjusted to between 8·2 and 8·7 by the addition of aqueous 0·4M-veronal solution (the concentration in the extract was about 0·04M).

Hydroxylamine solutions. These were prepared as described in the preceding paper.

Absorption spectra. All spectra were measured at 25±3° on a Unicam SP.500 spectrophotometer. Specially designed glass cells of 1 cm. optical-path length enabled measurements to be carried out on 0·6 ml. of solution.

Bleaching apparatus

Flash equipment. This was as described in the preceding paper, except that the optical-cell holder was modified so that alcohol at various temperatures could be passed through it. In this way solutions could be maintained at any desired temperature, measured by means of a copper–constantan thermocouple which was dipped into the rhodopsin solution. Condensation was prevented by circulating dry air through the interior of the apparatus. A further alteration to the optical-cell holder permitted the attachment of Ilford gelatin filters close to both optical faces; all radiation from the flash thus passed through these filters before irradiating the solution.

The spectral distribution of light reaching a solution was obtained by multiplying the relative spectral-energy curve of the flash (Robinson, 1954) by transmission spectra of the filters (measured on the Unicam SP.500). The relative energy distributions of the flash radiation after passing through the different colour filters and through one of the neutral filters used are shown in Fig. 1.

Normal white bleaching. When it was necessary to bleach solutions to completion, they were exposed for 15 min. at 25° to the light of a 15w, 230v lamp at a distance of 1 ft. (illumination ~25 ft.-candles).

Procedure. The experimental procedure was standardized as follows. (1) The absorption spectrum of the solution was

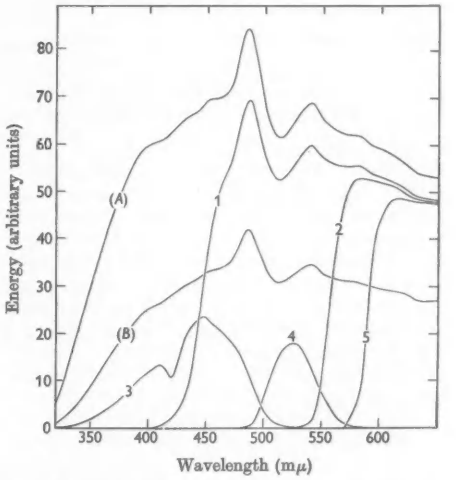


Fig. 1. Spectral-energy distribution of flash radiation after passing through some of the filters used. (*A*) Unfiltered (data of Robinson, 1954); (*B*) 'neutral' filter (nominal density of 0·31); 1, yellow, Ilford 102; 2, orange Ilford 201; 3, violet, Ilford 621; 4, green, Ilford 624; 5, red, Ilford 204.

first measured at 25° in a manner embodying a test of the thermal stability (Dartnall, 1952). (2) The solution was placed in the flash equipment and gradually cooled or warmed to the desired temperature. (3) The solution was flash-irradiated through the correct filter, brought as rapidly as possible to 25° and left for 1 hr. in darkness, after which time all thermal changes were complete (cf. the preceding paper). Subsequent operations were carried out at 25°. (4) The absorption spectrum was again measured. (5) The solution was finally exposed to 'normal' white light for 15 min. and the absorption spectrum of the fully bleached solution obtained. Difference spectra for the parent rhodopsin and for Fraction 2 could then be calculated by subtraction of the final fully bleached absorption spectrum from the initial and post-flash absorption spectra respectively (as illustrated in the preceding paper, Fig. 2).

Analysis of difference spectra. Fraction 2 contains P486 and frog rhodopsin, hence its difference spectrum is displaced from that of frog rhodopsin to an extent depending on the proportion of P486. Fig. 2 illustrates the wavelength shift produced at the long-wave 50% point when varying proportions of the P486 difference spectrum are added to that of frog rhodopsin. Thus all that is necessary when determining the composition of a Fraction 2 is to compare its difference spectrum with that of frog rhodopsin, measure the displacement and then read off the percentage of P486 from Fig. 2.

Quantities of photosensitive materials are expressed in terms of their maximum change in extinction when fully bleached ($\Delta E_{max.}$). When the difference spectra of P486 and frog rhodopsin, both with $\Delta E_{max.}$ of 50, are added the $\Delta E_{max.}$ of the resulting difference spectrum is not 100 but 98·4 (see preceding paper). According to the proportions in which these difference spectra are added, the resulting $\Delta E_{max.}$ may lie between 98·4 and 100·0.

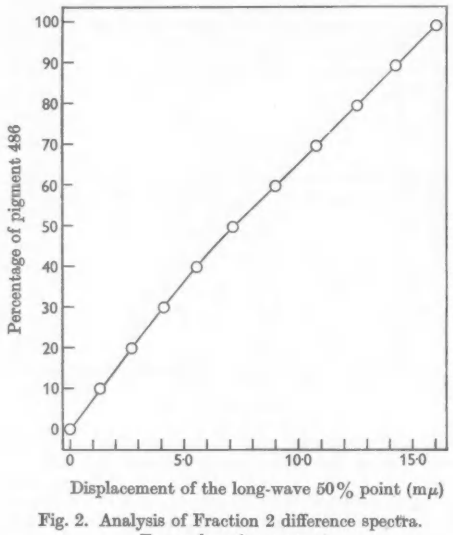


Fig. 2. Analysis of Fraction 2 difference spectra. For explanation see text.

Consequently, after determining the percentage of P486 in Fraction 2, a corresponding small correction must be applied to the $\Delta E_{max.}$ of the Fraction 2 difference spectrum to give the total photosensitive-pigment content.

$\Delta E_{max.}$ as a measure of photosensitive-pigment concentration. For a given visual pigment under a given set of conditions (e.g. pH, temperature etc.) $\Delta E_{max.}$ is proportional to $E_{max.}$ and hence to the visual-pigment concentration (cf. Bridges, 1957). However, when the quantities of pigments in a mixture are being compared a word of qualification is necessary, since alkaline indicator yellow and retinene oxime absorb slightly differently at the respective $\lambda_{max.}$ of frog rhodopsin and P486. This, however, has only a very small effect on the estimated proportions of these pigments in a mixture. Consequently, until the respective molar-extinction coefficients are known (Dartnall, 1955), the $\Delta E_{max.}$ values provide useful means of expressing concentrations in such mixtures.

RESULTS

Effect of flash intensity on the composition and yield of Fraction 2 produced at 25°

'Fraction 2' designates any material remaining in solution after flash-irradiation and which is thermally stable and photosensitive at 25°. If the

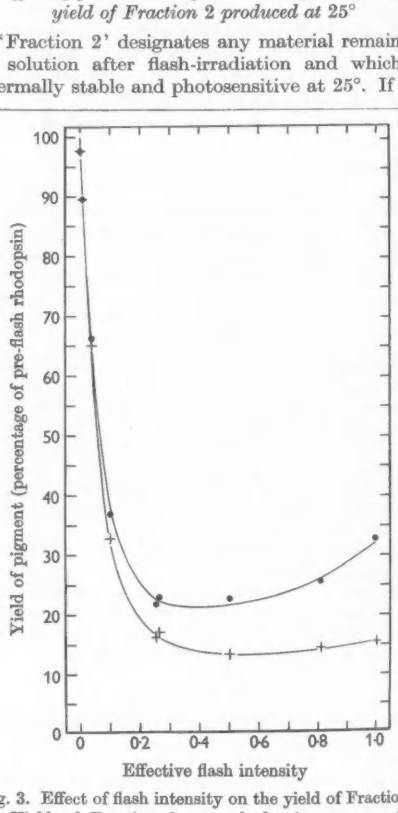


Fig. 3. Effect of flash intensity on the yield of Fraction 2. ●, Yield of Fraction 2; +, rhodopsin component of Fraction 2.

flash intensity is decreased by means of a filter to 1/1000 of the unfiltered value, Fraction 2 is found to be entirely rhodopsin and amounts to 97% of the pre-flash rhodopsin. Clearly, in this case 'Fraction 2' is merely unbleached rhodopsin. As the flash intensity is raised the yield of Fraction 2 decreases, as shown in Fig. 3, down to a minimum of about 22%, but thereafter rises again until at the full flash intensity it is about 33% of the pre-flash rhodopsin.

Some explanation of the abscissa of Fig. 3 is necessary here. The effective fraction of light transmitted by a filter depends on the light-absorbing properties of the photo-reacting material, in this case frog rhodopsin. Therefore, to obtain the effective reduction of light intensity by any filter, neutral or coloured, relative spectral-energy curves, such as are shown in Fig. 1, must be multiplied by the extinction curve (see end of Discussion) of pure frog rhodopsin (tabulated by Dartnall, 1953). The areas beneath each of the derived filtered curves must then be divided into the area beneath the unfiltered curve. The final result, called the effective flash intensity (EFI), is therefore equal to:

$$\frac{\int_{400}^{620} A_\lambda . E_\lambda . d\lambda}{\int_{400}^{620} A_\lambda . E_\lambda . T_\lambda . d\lambda},$$

where A_λ = relative spectral energy of xenon-tube emission at wavelength λ; E_λ = relative extinction of frog rhodopsin at wavelength λ; T_λ = percentage transmission of filter at wavelength λ.

In addition to the changes in yield of Fraction 2, the proportion of P486 (estimated as described in the Methods section) rose from 0% at low flash intensities to between 46 and 57% at full flash intensity, as shown in Fig. 4. The lower curve of Fig. 3 shows the changes in yield of the rhodopsin component only, and clearly indicates that although the proportion of rhodopsin in the mixture decreased, the actual yield rose slightly at EFI values above about 0·5. Thus although the 'Fraction 2' of low flash intensities probably consisted entirely of unbleached rhodopsin, that of higher flash intensities, in addition to containing flash-generated P486, contained also flash-generated rhodopsin.

To summarize: as the effective flash intensity is increased, the following changes in Fraction 2 are observed: (1) the total yield of photosensitive pigments decreases, passes through a minimum, then rises steadily until full flash intensity is reached; (2) the proportion of P486 increases; (3) after passing through a minimum, the yield of frog rhodopsin increases slightly.

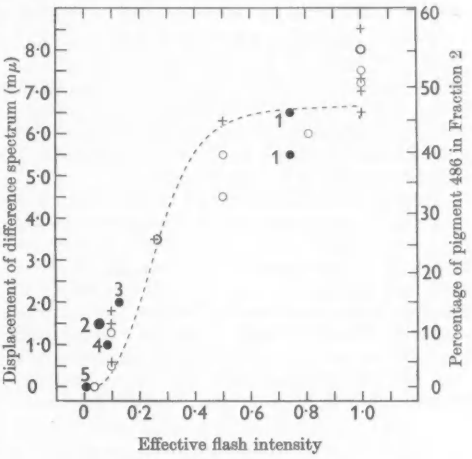


Fig. 4. Effect of flash intensity on the percentage of pigment 486 in Fraction 2. Solutions irradiated through neutral filters: ○, hydroxylamine-free solutions; +, solutions containing 0·01 M-hydroxylamine. Solutions irradiated through coloured filters: ●, hydroxylamine-free solutions. The number labelling each point denotes the use of one of the following colour filters: 1, yellow, Ilford 102; 2, orange, Ilford 201; 3, violet, Ilford 621; 4, green, Ilford 624; 5, red, Ilford 204. The broken line is the theoretical relationship between flash intensity and percentage of pigment 486 (see Discussion).

Effect of flash colour on the composition and yield of Fraction 2 produced at 25°

It was noted in the preceding paper that although the difference spectrum of Fraction 1 was the same for orange and unfiltered flashes, Fraction 2 produced by an orange flash was found to contain less P486 (i.e. its $\lambda_{max.}$ was at 500 compared with 494 mμ). If, however, the EFI for the orange filter is computed, and the result of this experiment is inserted into Fig. 4, it is apparent that the orange results fit the 'neutral' P486 (%)/EFI curve. Results of experiments with red, green, yellow and violet flashes similarly agree with the corresponding 'neutral' values, as illustrated. Again, in terms of the yield of photosensitive pigment in Fraction 2, no consistent wavelength effect could be detected, as shown by Fig. 5.

To summarize: the composition and yield of Fraction 2 produced by flashes of varying chromaticity is accounted for within experimental error on the basis that the coloured filters merely reduced the effective light intensity for the bleaching of rhodopsin.

Flash-irradiation in the presence of hydroxylamine

In the preceding paper the composition and yield of Fraction 2 produced at 25° by an unfiltered flash were found to be unaffected by hydroxylamine (0·01 or 0·03 M). Similarly, in the present work, hydroxylamine (0·01 M) was found to have no effect on the Fraction 2 produced by flashes of any intensity, as shown in Figs. 4 and 5.

Flash-irradiation of Fraction 2 at 25°. Fraction 2 produced in the presence of 0·01 M-hydroxylamine by a flash of EFI 0·10 contained 12·4 % of P 486. Exposure of this material to a second identical flash, and leaving for 1 hr. to permit completion of thermal changes, produced a Fraction 2 which now contained 22·4 % of P 486. Yet a third flash raised the P 486 to 44·0 %. At higher flash intensities this rise was still observed but was not so marked. Thus at full flash intensity, the first-flash Fraction 2 contained 49·8 % of P 486, whereas second- and third-flash Fractions 2 (in the presence of hydroxylamine) respectively contained 59·0 and 62·8 % of P 486. Hydroxylamine (0·01 M) was added to the solutions used in these experiments, so that after the decay of Fraction 1 in the first flash the solutions contained retinene oxime and Fraction 2. Thus the effect of a second flash was not complicated by regeneration of rhodopsin from irradiated indicator yellow. This is demonstrated by the fact that first-, second- and third-flash Fractions 2 formed in a hydroxylamine-free solution exposed to full-intensity flashes contained respectively 50·5, 41·6 and 39·6 % of P 486.

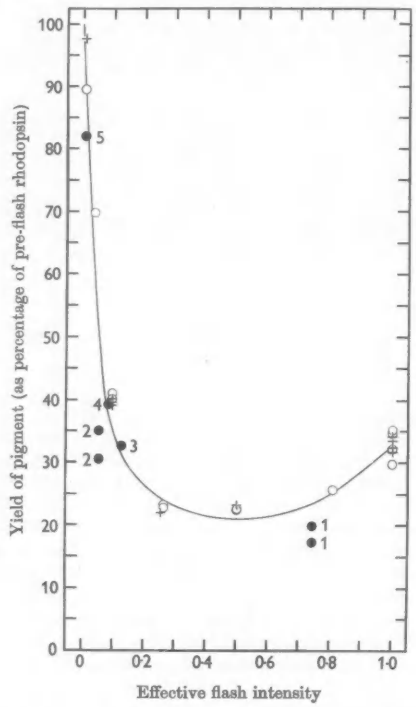


Fig. 5. Comparison between the yields of Fraction 2 produced in solutions (with or without hydroxylamine) irradiated through neutral or coloured filters. Solutions irradiated through neutral filters: ○, hydroxylamine-free solutions; +, solutions containing 0·01 M-hydroxylamine. Solutions irradiated through coloured filters: ●, hydroxylamine-free solutions; the points are numbered as in Fig. 4.

Flash-irradiation at different temperatures

The preceding results were obtained by irradiating solutions at 25°. Experiments with full-intensity flashes were also carried out at temperatures from +40° to −41°. The yield of Fraction 2, after leaving the irradiated solutions for 1 hr. at 25°, was found to increase by between 4 and 10 % for every 10° fall in temperature. This is illustrated by the absorption spectra shown in Fig. 6. In this Figure, the curves labelled (*b*) represent absorption spectra of solutions measured after irradiation at a number of different temperatures and then leaving for 1 hr. at 25°. The yield of Fraction 2, as measured by the maximum fall of extinction when the solutions were fully bleached (curves *c*), is seen to rise from about 32 % of the pre-flash rhodopsin at +40° to about 53 % at −41°. The analysis of these and other experiments is given in Table 1, and shows that although the yield of Fraction 2 depended on the temperature, there were no significant changes in the composition of this material.

The influence of temperature on the yield of Fraction 2 does not appear to be linked in any way to accompanying changes in viscosity. Thus the yield of Fraction 2 produced at +8° in a viscous solution containing 65 % (v/v) of glycerol is practically identical with that produced at the same temperature in a solution without glycerol.

DISCUSSION

Only the reactions occurring after irradiation by a single flash will be considered here. The effect of subsequent flashes is complicated by the fact that one is no longer dealing with a single pigment, but with a mixture of two pigments.

Fraction 2 present in solution after irradiation at very low flash intensities was found to consist entirely of rhodopsin, and this rhodopsin was probably merely unbleached material. If this were true of the rhodopsin component of Fraction 2 produced by a flash of any intensity, one would

expect that the curve relating its yield to the flash intensity would asymptotically approach the zero line according to the equation

$$C = a e^{-kIt},$$

where a = initial rhodopsin (= 100), C = rhodopsin yield, I = effective flash intensity, t = duration of photochemical reaction, k = constant. However, this equation does not describe the observed rhodopsin concentrations, as is apparent from the lower curve of Fig. 3. Here, the rhodopsin yield, after passing through a minimum, actually rises as the highest flash intensity is approached. This increase can only be due to the presence of rhodopsin

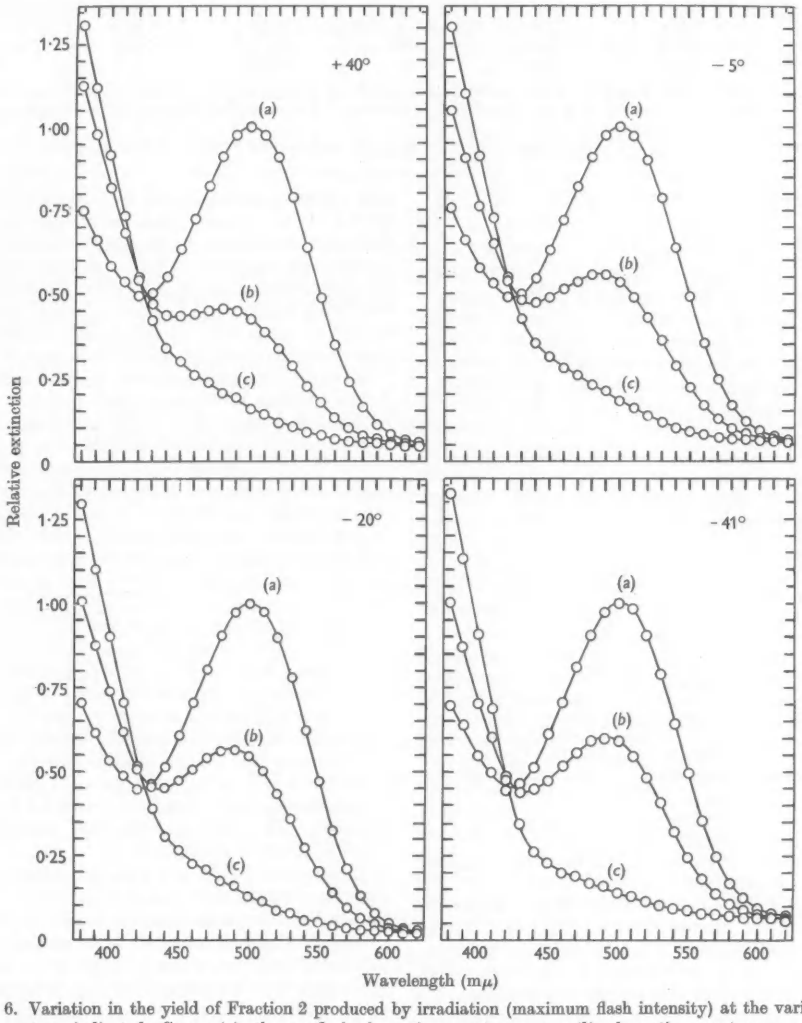


Fig. 6. Variation in the yield of Fraction 2 produced by irradiation (maximum flash intensity) at the various temperatures indicated. Curves (*a*), the pre-flash absorption, spectra; curves (*b*), absorption spectra measured after flash-irradiating the solution at the given temperature, then leaving it for 1 hr. at 25°; curves (*c*) are absorption spectra of the fully bleached solutions, i.e. after exposure for 15 min. (at 25°) to a 15 w lamp. The maximum loss of extinction from curve (*b*) to curve (*c*) is a measure of the amount of Fraction 2. All spectra were measured at 25°. Pre-flash absorption spectra have been scaled so that $E_{500\,m\mu} = 1{\cdot}00$.

Table 1. *Effect of temperature on the yield and composition of Fraction 2*

$\Delta E_{max.}$ (1 cm.)	Glycerol content* (%, v/v)	Temperature	Yield of Fraction 2† (% of original rhodopsin)	Pigment 486 in Fraction 2 (%)
0·575	50	+40°	31·83	54·0
0·578	50	+25	31·92	49·5
0·401	0	+25	34·69	49·5
0·415	65	+8	41·81	53·5
0·418	0	+8	41·84	50·0
0·534	50	−5	43·90	46·0
0·550	50	−20	48·78	50·0
0·414	65	−41	53·02	47·1

* Solutions were adjusted to pH 8·2–8·7 by addition of one-tenth of the extract volume of aqueous solutions of saturated sodium borate (for solutions with no glycerol) or 0·4M-sodium 5:5′-diethylbarbiturate (for solutions containing glycerol).

† Measured after bringing the temperature of the solution to 25° and leaving for 1 hr. at that temperature.

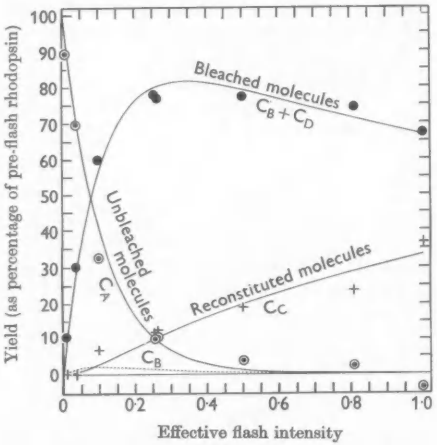


Fig. 7. Variation with flash intensity of the three types of substance present in a solution of frog rhodopsin after exposure to a flash. ⊙, Unbleached molecules (the curve represents C_A); ●, bleached molecules (the curve represents $C_B + C_D$); +, reconstituted molecules (the curve represents C_C). The broken line represents C_B, the concentration of the hypothetical intermediate B, at the end of the photochemical reaction.

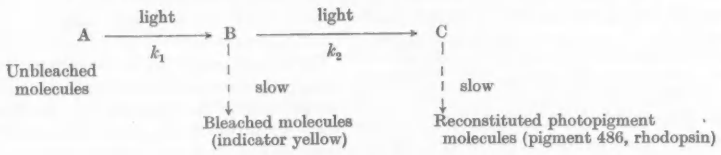
reconstituted by the flash. It is therefore suggested that the curve relating flash intensity to the quantity of Fraction 2 produced (Fig. 3) is the sum of two curves. One represents unbleached rhodopsin, and diminishes with rising flash intensity according to the above equation. The other, representing the mixture of reconstituted rhodopsin and pigment 486, must increase with rising flash intensity, since the total Fraction 2 increases.

Experimentally, it is not possible to arrive at an estimate of the proportion of unbleached rhodopsin in any given Fraction 2. If, however, it is assumed that pigment 486 represents a constant proportion (47%) of the reconstituted photopigments at all flash intensities, then it is a straightforward matter to calculate the yield of reconstituted material at any particular intensity. Values for the unbleached rhodopsin may then be obtained which, as shown in Fig. 7, agree with corresponding values calculated from the above equation, when $kt = 9$. Therefore the assumption appears to be justified.

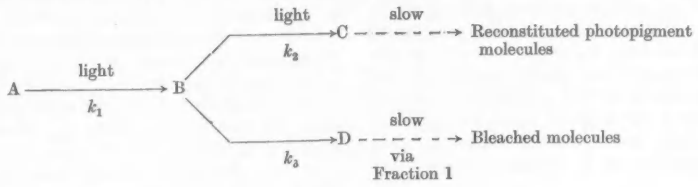
The increase with rising flash intensity of the reconstituted photopigment (Fig. 7) suggests that it is formed by the irradiation of some substance which is an intermediate in the sequence of reactions after absorption of a single light quantum by the rhodopsin molecule, as shown in Scheme I. In this scheme, A represents the original rhodopsin and B represents the intermediate state convertible by light into another state C. Both B and C are slowly converted into bleached molecules (indicator yellow ultimately) and reconstituted photopigment molecules respectively. The independence of the composition of Fraction 2 on the wavelength of the irradiating light would make it likely that conversion of C into photosensitive material is slow compared with the duration of the flash. Were this not so, long-wavelength flashes would produce Fractions 2 rich in pigment 486 and short-wavelength flashes would produce Fractions 2 poor in pigment 486 (relative to the corresponding 'neutral' flash composition).

If the yields of A, B and C are calculated from the kinetics of this Scheme (assuming all reactions to be first-order) the results describe the observed yields of bleached and reconstituted molecules in Fig. 7. A better fit of these results is, however, obtained by assuming that the intermediate 'B' decomposes at a rate comparable with the duration of the flash, as shown in Scheme II. If the kinetics of these reactions are first-order we have

$$-\frac{dC_A}{dt} = k_1 I C_A \quad \text{or} \quad C_A = a e^{-k_1 I t},$$



Scheme I



Scheme II

where $a = C_A + C_B + C_C + C_D = 100$, C_A, C_B, C_C and C_D represent the percentage yields of A, B, C and D respectively. Similarly,

$$\frac{dC_C}{dt} = k_2 I C_B$$

and

$$\frac{dC_D}{dt} = k_3 C_B.$$

Therefore

$$\frac{dC_B}{dt} = -\frac{dC_A}{dt} - \left[\frac{dC_C}{dt} + \frac{dC_D}{dt}\right].$$

From the above equations

$$\frac{dC_B}{dt} = k_1 I e^{-k_1 I t} - [k_2 I + k_3] C_B.$$

This may be integrated to

$$C_B = \frac{a k_1}{\frac{k_3}{I} + k_2 - k_1} [e^{-k_1 I t} - e^{-(k_2 I + k_3)t}]$$

(cf. Rutherford, Chadwick & Ellis, 1930). Since:

$$\frac{C_C}{C_D} = \frac{k_2 I}{k_3},$$

therefore

$$C_D = \frac{a - (C_A + C_B)}{1 + (k_2 I / k_3)}.$$

Knowing C_A, C_B and C_D it is then possible to calculate C_C.

When all thermal changes have ceased after flash-irradiation, the yield of bleached molecules is given by $(C_B + C_D)$, and of reconstituted molecules by C_C. As shown in Fig. 7, the curves relating these two quantities to the light intensity are in satisfactory agreement with the experimental points when $k_1 t = 9$, $k_2 t = 9$ and $k_3 t = 18$. The broken curve of Fig. 4, representing the calculated percentage of pigment 486 in Fraction 2, is similarly not incompatible with the experimental observations. If it is assumed that the rates of the photochemical reactions are independent of temperature, the rate of conversion of B into D has a temperature coefficient of between 1·14 and 1·08.

It should be noted that if the extinction curve of frog rhodopsin changes with temperature in the same way as that of cattle rhodopsin, then the effective flash intensity would be 1·09 at −45°. (This has been calculated from the data published by Wald, Durell & St George, 1950.) However, if this value is inserted into the equations shown above, the yield of Fraction 2 increases only by about 7% from +23° to −45°. As shown in Table 1, the observed increase from +25° to −41° was about 60%.

At present the identity of the hypothetical state B is unknown. Clearly it cannot be indicator yellow (see preceding paper), and furthermore the absence of any consistent wavelength effect in the coloured-flash experiments illustrated in Figs. 4 and 5 shows that the photoreacting intermediate B had an absorption spectrum identical with or very similar to that of frog rhodopsin. Substance B may be the result of the primary photochemical event, i.e. it is possibly an electronically excited state of the rhodopsin molecule. Again, it may be a new substance of the type found in the immediate post-flash period by Wulff *et al.* (1958). Were the latter the case, there need be no conflict with the conclusions of the coloured-flash experiments, for the light-absorbing properties of these substances differed little from those of the parent rhodopsin.

Effective flash intensity

It might be expected that the light-absorbed curve rather than the extinction curve of rhodopsin should be used to estimate the effective fraction of

light transmitted by the filters. The light absorbed at any wavelength λ may be calculated from the expression

$$I_{abs.} = I_{inc.}(1-10^{-E_\lambda}),$$

where $I_{abs.}$ = light absorbed, $I_{inc.}$ = light incident on the solution, E_λ = extinction at wavelength λ. If $E_\lambda \leqslant 0{\cdot}05$ this expression approximates to $I_{abs.} = 2{\cdot}303\ I_{inc.}E_\lambda$ (cf. Dartnall, 1957). Since $E_\lambda = \alpha_\lambda cl$ (where α_λ = extinction coefficient at wavelength λ; c = concentration; l = optical path length), it follows that E_λ is proportional to the number of chromophores in solution, and hence only when $E_\lambda \leqslant 0{\cdot}05$ is the light absorbed per molecule constant for a given value of $I_{inc.}$. The amount of light absorbed per molecule progressively diminishes as E_λ is raised above about 0·05.

Under the present experimental conditions, however, the yield of Fraction 2 produced by flash-irradiation of rhodopsin solutions was independent of the parent rhodopsin concentration and hence of the extinction of those solutions (Fig. 8). The $\Delta E_{max.}$ of these solutions ranged from about 0·3 to 1·3 yet the yield of Fraction 2 was always constant. It must therefore be accepted that, under the present experimental conditions, the light absorbed per rhodopsin molecule was constant over the range of extinctions tested. Therefore, since the light absorbed was proportional to the extinction, it is the extinction curve of rhodopsin which is used in assessing the 'effective flash intensity'.

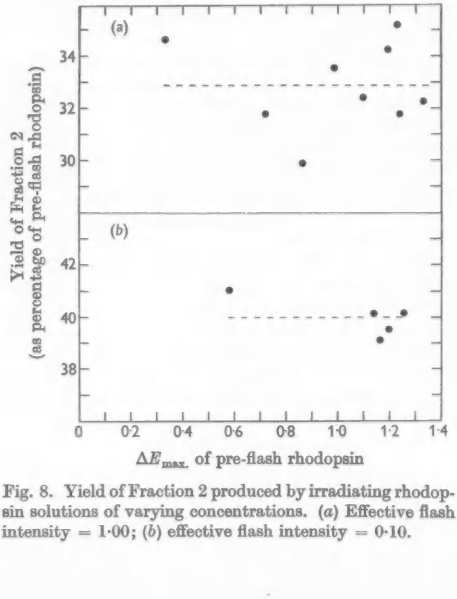


Fig. 8. Yield of Fraction 2 produced by irradiating rhodopsin solutions of varying concentrations. (*a*) Effective flash intensity = 1·00; (*b*) effective flash intensity = 0·10.

SUMMARY

1. Alkaline (pH 8·2–8·7) aqueous digitonin solutions of frog rhodopsin were exposed at different temperatures to intense 4 msec. flashes from a 400 J xenon-filled discharge tube. At all temperatures below 0° the solutions contained 50 or 65 % (v/v) of glycerol to prevent freezing. Some experiments were also carried out at higher temperatures on solutions which contained glycerol.

2. Exposing a solution at any temperature to a full-intensity flash and leaving for 1 hr. in darkness at 25° gives rise to a thermally stable photosensitive residue (Fraction 2) composed of the parent rhodopsin and a new pigment with $\lambda_{max.}$ at 486 mμ (pigment 486 or P 486).

3. The effects of altering flash intensity and colour on the composition and yield of Fraction 2 produced in solutions irradiated at 25° were investigated. If the flash intensity was reduced to about $\frac{1}{1000}$ of its unfiltered value, 'Fraction 2' amounted to about 97 % of the pre-flash rhodopsin and contained no P 486. This material was probably unbleached rhodopsin. As the flash intensity was raised, the yield of Fraction 2 steadily diminished to a minimum of about 22 % of the pre-flash rhodopsin, but thereafter rose to the full flash-intensity level of about 33 %.

4. At the same time, the proportion of P 486 in Fraction 2 rose from 0 % at low flash intensities to between 46 and 57 % at the maximum intensity. However, although the proportion of rhodopsin in Fraction 2 exhibited a corresponding decrease, the actual yield, after passing through a minimum, rose slightly towards the higher flash intensities.

5. The composition and yield of Fraction 2 produced in solutions irradiated at 25° by flashes of varying chromaticity is accounted for on the basis that the coloured filters merely decreased the effective light intensity for the bleaching of rhodopsin.

6. Hydroxylamine (0·01 M) had no effect on the composition and yield of Fraction 2 produced in solutions irradiated at 25° by flashes of any intensity.

7. If a solution were exposed, at 25°, to a number of flashes at intervals of about 1½ hr. the Fraction 2 produced by each successive flash was found to contain a progressively increasing proportion of P 486.

8. In solutions irradiated at various temperatures with full-intensity flashes and left for 1 hr. in darkness at 25°, the yield of Fraction 2 increased by between 4 and 10 % for every 10° drop in temperature between +40° and −41°. The changing viscosity of these solutions did not appear to be responsible for this effect. No significant alteration in the percentage of P 486 was observed.

9. It is suggested that Fraction 2 is made up of unbleached parent rhodopsin and a reconstituted constant-composition mixture of rhodopsin and P486. This reconstituted material is produced by the irradiation of an intermediate substance which is unstable even at $-40°$.

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Adenylic Deaminase of Pea Seeds

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Adenylic deaminase catalyses the deamination of adenosine 5′-monophosphate with the formation of inosine 5′-monophosphate and ammonia. The enzyme was first reported in extracts from rabbit muscle by Schmidt (1928), who showed that it was specific for adenosine 5′-monophosphate as substrate and was distinct from adenosine deaminase. Kalckar (1947) obtained active deaminase preparations from rabbit muscle after precipitating myosin and fractionating the enzyme with ammonium sulphate. Nikiforuk & Colowick (1956) purified adenylic deaminase some 40-fold from crude bicarbonate extracts of rabbit muscle and studied a number of the properties of the enzyme. Separation from myosin and further purification enabled Lee (1957) to crystallize the rabbit-muscle enzyme.

Adenylic deaminase has been found in several other animal tissues such as heart muscle, liver and kidney but the activities are lower than in skeletal muscle (Lee, 1957). The enzyme occurs as a complex with myosin (Kalckar, 1947; Humphrey & Webster, 1951).

The occurrence of the enzyme in a higher-plant tissue was investigated. In the present investigation adenylic deaminase was found in a particulate fraction from pea seeds and a preparation free from interfering enzymes was obtained. A number of properties of the pea-seed adenylic deaminase were studied.

EXPERIMENTAL

Special chemicals. Adenine, adenosine, adenosine 5′-monophosphate (AMP), adenosine diphosphate, adenosine triphosphate, inosine 5′-monophosphate (IMP) and diphosphopyridine nucleotide were obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A., triphosphopyridine nucleotide and *p*-hydroxymercuribenzoate from Sigma Chemical Co., St Louis, Mo., U.S.A., and cysteine hydrochloride from British Drug Houses Ltd.

Preparation of 5′-adenylic acid deaminase from pea seeds. Preliminary experiments showed that the adenylic-deaminase activity was associated with a particulate fraction in pea seeds and the following preparative procedure was adopted. Pea seeds (*Pisum sativum* L., var. Laxton's Progress) were soaked overnight in water and 50 g. of the soaked seeds was blended for 3 min. at 4° with 50 ml. of 40 mM-$NaHCO_3$. The homogenate was squeezed through muslin, centrifuged at 500 *g* for 5 min. and the supernatant centrifuged at 20 000 *g* for 30 min. The precipitate from the high-speed centrifuging was suspended in 10 ml. of water, centrifuged at 20 000 *g* for 30 min. and the resultant precipitate was dispersed in water to give a final volume of 5 ml.

The light-green enzyme preparation could be stored at $-15°$ for 5 weeks with 10% loss of deaminase activity. Heating at 60° for 5 min. resulted in 95% loss of activity. No phosphatase activity was detected when samples of reaction mixtures containing the enzyme preparation and AMP or IMP were examined for the formation of inorganic phosphate or, on paper chromatograms, for the production of adenosine or inosine.

Reaction mixtures. The reaction mixtures were maintained at 30°. The determination of enzyme activity was a

modification of the method described by Kalckar (1947). For the assay of 5′-adenylic deaminase the following components were mixed: 1·0 μmole of AMP, 100 μmoles of Li_2SO_4, 24 μmoles of succinic acid buffer (brought to pH 6·2 with NaOH) and 0·05 ml. of enzyme (containing approx. 0·4 mg. of protein N); total volume, 0·25 ml. The time of incubation was 30 min. Under these experimental conditions there was not more than 40% conversion of AMP into IMP and the rate of reaction was approximately constant during the incubation period. Control reaction mixtures in which AMP was replaced by IMP were always used.

In studying the effect of inhibitors the reaction was initiated by the addition of AMP after the other components of the reaction mixture had been incubated for 15 min. at 30°.

Samples of the reaction mixtures were inactivated by the addition of perchloric acid (final concn. 3·3%, w/w) for spectrophotometric and inorganic phosphate analyses or by the addition of 2 vol. of ethanol for chromatographic studies. After inactivation the samples were centrifuged at 1000 *g* for 5 min.

Estimation of adenosine monophosphate and inosine monophosphate. Extracts of the reaction mixtures in perchloric acid soln. were diluted with 30 vol. of water and the extinction at 265 mμ was measured in a Beckman model DU spectrophotometer. Solutions of AMP and IMP were standardized with a molar-extinction index of $15{\cdot}4 \times 10^3$ at 259 mμ and pH 7·0 for AMP (Bock, Ling, Morell & Lipton, 1956) and $12{\cdot}2 \times 10^3$ at 248·5 mμ and pH 6·0 for IMP (Beaven, Holiday & Johnson, 1955). From these solutions, calibrations were prepared for the extinction at 265 mμ of the nucleotides in perchloric acid soln. The results, which are calculated from the decrease in extinction at 265 mμ, are expressed as μmoles of IMP formed per reaction mixture.

Estimation of inorganic phosphate. Extracts of the reaction mixtures in perchloric acid soln. were analysed for inorganic orthophosphate by the method of Allen (1940) as modified by Turner (1957).

Chromatography. Nucleotides in the ethanolic extracts of the reaction mixtures were separated and detected on paper chromatograms as previously described (Turner & Turner, 1958). Either *iso*butyric acid–aq. NH_3 soln. (sp.gr. 0·88)–water (66:1:33, by vol.) (Pabst Laboratories, 1956) or Na_2HPO_4–NaH_2PO_4 buffer, pH 6·8 (0·1 M, 50 ml.)–$(NH_4)_2SO_4$ (30 g.)–propan-1-ol (1 ml.) (Pabst Laboratories, 1956) was used as solvent.

For further identification of IMP the nucleotide was eluted from the spot area with 3 ml. of 50 mM-phosphate (KH_2PO_4–Na_2HPO_4) buffer, pH 7 (Kirkland & Turner, 1959). Appropriate areas of paper were taken for blank values.

Estimation of protein nitrogen. Protein in the enzyme preparation was precipitated by 5% (w/v) trichloroacetic acid and washed twice with 1% (w/v) trichloroacetic acid. Total N in the residue was determined by the micro-Kjeldahl procedure.

RESULTS

5′-Adenylic acid-deaminase activity. The deamination of AMP by the pea-seed adenylic deaminase is shown in Fig. 1. In the experiment illustrated 11·0 μmoles of AMP were added as substrate and 10·8 μmoles of IMP were formed. In experiments of this type 95–98% of added AMP could be accounted for as IMP.

Although phosphatase activity was present in the crude homogenate from pea seeds, it was not detected by the formation of inorganic phosphate, adenosine or inosine when the washed particle preparation was incubated in reaction mixtures containing AMP or IMP. There was no change in extinction during the incubation of reaction mixtures containing IMP and the adenylic-deaminase preparation. These observations indicated that the enzyme preparation was free from interfering enzymes.

IMP was isolated from reaction mixtures by applying ethanolic extracts to Whatman no. 3MM papers which were developed in the *iso*butyric acid–aq. NH_3 soln.–water solvent. The area corresponding to IMP was cut out and eluted. The absorption spectrum of the eluted material was identical to that of IMP.

Effect of anions. In preliminary experiments active deaminase preparations were obtained by washing the particle fraction from pea seeds with 20% saturated $(NH_4)_2SO_4$, pH 6·0. There was no activity in these preparations after dialysis for 2·5 hr. at 4° against five changes of water, but the preparation showed activity on the addition of $(NH_4)_2SO_4$ (final concn. 0·2 M).

The deaminase preparation used in the present investigation was normally prepared by washing the particulate fraction with water and possessed negligible or no activity in the absence of added salts. The effects of the addition of various salts on the deaminase activity are shown in Table 1. The

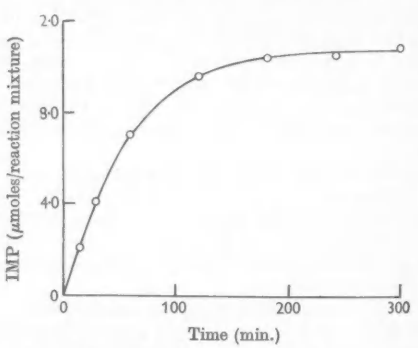

Fig. 1. 5′-Adenylic acid-deaminase activity as measured by IMP formation. The reaction mixture contained 11·0 μmoles of AMP, 1 m-mole of Li_2SO_4, 0·24 m-mole of succinate buffer, pH 6·2, and 0·5 ml. of enzyme (containing 4·2 mg. of protein N); total volume, 2·5 ml. Temp. 30°.

sulphates of the cations tested were more effective than the chlorides but the nature of the cation also had some effect. The maximum increase was given by $(NH_4)_2SO_4$ and Li_2SO_4 in relatively high final concentration (0·4 M). Citrate produced a good stimulation of deaminase activity but acetate did not increase the activity in any of the concentrations tried. Increasing the salt concentration above the optimum led to a decrease in the rate of reaction.

Effect of pH. The effect of pH on the activity of pea-seed adenylic deaminase is shown in Fig. 2. With succinate buffer the optimum pH was 6·0–6·2, but with 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–acetic acid buffer there was a broader optimum from approx. pH 5·6 to 6·8.

Effect of substrate concentration. The effect of concentration of AMP on the initial reaction velocity of the adenylic deaminase is shown in Fig. 3. With the method of Lineweaver & Burk (1934) it was calculated from these results that the Michaelis–Menten constant (K_m) for AMP was $1·3 \times 10^{-2}$ M.

Effect of inhibitors. The effects of several inhibitors on adenylic-deaminase activity are shown in Table 2. The enzyme was inhibited by phosphate and fluoride and also by the metal ions Zn^{2+}, Cu^{2+} and Hg^{2+}. The enzyme was sensitive to the sulphydryl reagents *p*-hydroxymercuribenzoate and iodoacetate. Ethylenediaminetetra-acetate inhibited the reaction. Cysteine had no effect.

Table 1. *Effect of anions on adenylic-deaminase activity*

Reaction mixtures contained 1·05 μmoles of AMP, 24 μmoles of succinate buffer, pH 6·2, 0·05 ml. of enzyme (containing 0·42 mg. of protein N); total volume, 0·25 ml. Salts were added as shown. Time of incubation, 30 min. Temp. 30°. The results are expressed as the increased formation of IMP (μmole/reaction mixture) above the control figure (0·05 μmole) obtained in a reaction mixture containing no added salt.

	Final concn. (M)			
Addition	0·01	0·1	0·4	0·8
$(NH_4)_2SO_4$	0	0·24	0·37	0·22
Li_2SO_4	0	0·07	0·34	0·11
Na_2SO_4	0	0	0·29	0·13
$MgSO_4$	0	0·25	0·19	0·03
NH_4Cl	0	0·03	0·20	0·09
NaCl	0	0·01	0·14	0·06
$MgCl_2$	0·05	0·18	0	0
Sodium citrate	0·01	0·25	0·16	0·06
Sodium acetate	0	0	0	0

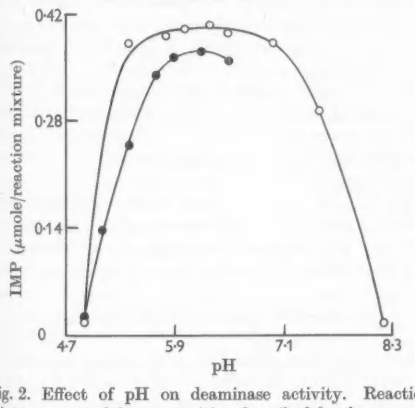

Fig. 2. Effect of pH on deaminase activity. Reaction mixtures were of the composition described for the assay of 5′-adenylic acid deaminase. Succinic acid (24 μmoles)–NaOH (●) and tris (25 μmoles)–acetic acid (○) buffers of the required pH were used. Time of incubation, 30 min. Temp. 30°.

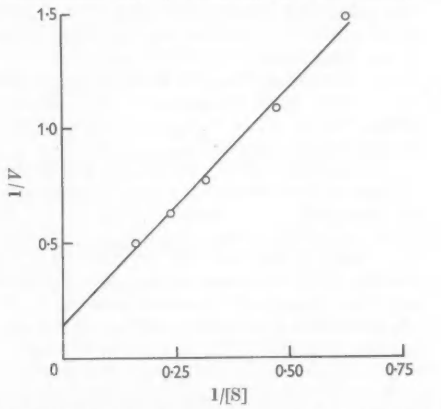

Fig. 3. Effect of substrate concentration on deaminase activity. Substrate concentration, [S], is expressed as μmole of AMP/ml.; initial velocity, V, is expressed as μmoles of IMP formed/ml. per 30 min. Reaction mixtures were of the composition described for the assay of deaminase activity with the AMP concentration varied as shown. Time of incubation, 30 min. Temp. 30°.

Table 2. *Effect of inhibitors on adenylic-deaminase activity*

Reaction mixtures were of the composition described for the assay of 5′-adenylic acid deaminase. Inhibitors were added as shown. Time of incubation, 30 min. Temp. 30°.

Inhibitor	Final concn. (mM)	Inhibition (%)
NH_4F	1	38
KH_2PO_4	8	76
$ZnSO_4$	5	23
$CuSO_4$	1	100
$HgCl_2$	0·01	100
p-Hydroxymercuribenzoate	0·2	100
Iodoacetate	10	39
Ethylenediaminetetra-acetate	100	60
Cysteine	10	0

Specificity of the adenylic-deaminase preparation. The pea-seed-deaminase preparation was specific for AMP as substrate. There was no reaction when AMP was replaced by adenine, adenosine, adenosine diphosphate, adenosine triphosphate, diphosphopyridine nucleotide or triphosphopyridine nucleotide in reaction mixtures of the composition described for the assay of 5′-adenylic deaminase.

DISCUSSION

This investigation has established that active 5′-adenylic-deaminase preparations free from interfering enzymes may be readily obtained from pea seeds. From the range of compounds tested the enzyme was specific for AMP as substrate and had no action on adenine, adenosine, adenosine diphosphate or adenosine triphosphate.

From the data illustrated in Fig. 1 it can be concluded that the reaction was essentially irreversible and proceeded virtually to completion. Kalckar (1947) was unable to detect any reversal of the reaction in the presence of a relatively high concentration of NH_4^+ ions, and similar results were obtained by Lee (1957).

The stimulatory effect of ions on the pea-seed deaminase was very pronounced and most preparations possessed no detectable activity in the absence of added salts. Ammonium sulphate, lithium sulphate and sodium sulphate were the most effective of the salts tested and the effect appeared to be largely due to the anion, as ammonium chloride and sodium chloride gave substantially less stimulation. The addition of sodium citrate increased the rate of reaction but no detectable increase over the control value was obtained with sodium acetate. Humphrey & Webster (1951) found that the deaminase activity of preparations of rabbit-muscle myosin was accelerated in the presence of 10 mM-calcium chloride or -magnesium chloride. The effect of anions on rabbit-muscle deaminase was studied by Nikiforuk & Colowick (1956). These authors found that citrate, chloride, acetate and lactate stimulated deaminase activity but sulphate had no effect. The enzyme preparation used by Nikiforuk & Colowick was active without added salts and the maximum stimulation of activity obtained (approximately 150%) was much lower than that obtained with pea-seed deaminase. The salt concentration employed by these authors was lower than that found to be optimum in the present investigation. No effect of chloride, citrate or acetate was found by Lee (1957), with the crystalline deaminase from rabbit muscle. The optimum pH for pea-seed deaminase in succinate buffer (pH 6·0–6·2) agrees well with the figures obtained for the rabbit-muscle enzyme. Kalckar (1947) and Nikiforuk & Colowick (1956) reported a value of 5·9, and Lee (1957) found that the optimum pH for the crystalline rabbit-muscle enzyme was 6·4 in succinate buffer. The K_m for AMP with the pea-seed deaminase was $1{\cdot}3 \times 10^{-2}$ M and this value is considerably greater than the K_m of $6{\cdot}0 \times 10^{-5}$ M for the rabbit-muscle enzyme reported by Nikiforuk & Colowick (1956), but is closer to that of $1{\cdot}4 \times 10^{-3}$ M reported by Lee (1957) for the crystalline enzyme.

In the present investigation it was found that the deaminase was inhibited by fluoride and inorganic orthophosphate and this agrees with observations with the enzyme from animal tissues (Nikiforuk & Colowick, 1956; Lee, 1957). Zn^{2+} ions inhibited the pea-seed deaminase and this is in agreement with the results of Lee but not with those of Nikiforuk & Colowick, who found no inhibition by Zn^{2+} ions. Hg^{2+} and Cu^{2+} ions strongly inhibited the plant deaminase. The sensitivity of the pea-seed enzyme to Hg^{2+} ions suggested that sulphydryl groups were essential for the enzyme activity and this was confirmed by the inhibition by *p*-hydroxymercuribenzoate and iodoacetate. Neither Nikiforuk & Colowick nor Lee found iodoacetate inhibition, although Lee observed inhibition by *p*-mercuribenzenesulphonic acid. Ethylenediaminetetra-acetate inhibited the enzyme used in the present investigation. This suggests that the pea-seed deaminase may have a requirement for a bivalent metal ion and this is supported by the fluoride inhibition. Nikiforuk & Colowick and Lee did not, however, observe inhibition by ethylenediaminetetra-acetate.

SUMMARY

1. 5′-Adenylic acid deaminase was found in a particulate fraction obtained from pea-seed extracts. A preparation free from interfering enzymes was obtained.

2. The enzyme was essentially inactive in the absence of an added salt. The sulphates of univalent cations were most effective in stimulating the reaction; the effect was largely due to the anion component.

3. The effects of pH and concentration of substrate on adenylic-deaminase activity were studied. Under the experimental conditions the Michaelis–Menten constant for adenosine monophosphate was $1{\cdot}3 \times 10^{-2}$ M.

4. The enzyme was inhibited by fluoride, inorganic phosphate, Zn^{2+} ions, Cu^{2+} ions, Hg^{2+} ions, *p*-hydroxymercuribenzoate, iodoacetate and ethylenediaminetetra-acetate.

5. The enzyme was specific for adenosine monophosphate.

6. The properties of the pea-seed adenylic deaminase have been considered in relation to those of the enzyme from rabbit muscle.

The work described in this paper was carried out as part of the joint research programme of the Division of Food Preservation, C.S.I.R.O., and of the Botany School, University of Sydney. The authors wish to express their indebtedness to Miss S. K. Harris and Miss N. J. Eames for technical assistance; and to Dr J. R. Vickery, Chief, Division of Food Preservation, and Professor R. L. Crocker, Botany School, University of Sydney, in whose laboratories the work was carried out.

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Inosine 5′-Phosphate Dehydrogenase of Pea Seeds

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Inosine 5′-phosphate (hypoxanthine riboside 5′-phosphate) has been implicated as a precursor of nucleic acid purines by the work of Greenberg (1952) and Buchanan & Schulman (1953). Adenosine 5′-phosphate may be formed from inosine 5′-phosphate by way of adenylosuccinate (Carter & Cohen, 1955, 1956; Lieberman, 1956). Investigations have shown that a derivative of xanthosine (xanthine riboside) is an intermediate in the formation of guanosine 5′-phosphate from inosine 5′-phosphate by extracts of rabbit bone marrow and pigeon liver (Abrams & Bentley, 1955*a*, *b*; Lagerkvist, 1955, 1958). The first reaction involved, the enzymic conversion of inosine 5′-phosphate into xanthosine 5′-phosphate, was demonstrated by Abrams & Bentley (1955*a*) in a soluble extract from rabbit bone marrow and by Gehring & Magasanik (1955) in fractionated extracts of *Aerobacter aerogenes*; diphosphopyridine nucleotide was reduced in the reaction. The enzyme involved, inosine 5′-phosphate dehydrogenase, was purified from extracts of *A. aerogenes* by Magasanik, Moyed & Gehring (1957).

In the present investigation inosine 5′-phosphate dehydrogenase was found in a higher plant and some properties of the enzyme from pea seeds were studied. It was shown previously that adenosine 5′-phosphate is deaminated to inosine 5′-phosphate by an adenylic deaminase present in pea-seed extracts (Turner & Turner, 1961).

MATERIALS AND METHODS

Substrates. Inosine, inosine 5′-phosphate, guanosine 5′-phosphate and diphosphopyridine nucleotide (DPN) were obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A., xanthosine from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., triphosphopyridine nucleotide (TPN) from Sigma Chemical Co., St Louis, Mo., U.S.A., and glutathione (GSH) from C. F. Boehringer und Soehne, Mannheim, Germany.

Preparation of inosine 5′-phosphate dehydrogenase from pea seeds. A crude extract was prepared from dried pea seeds (*Pisum sativum* L., var. Canners' Perfection) as previously described (Turner, 1957) except that toluene was omitted. The crude extract was centrifuged at 20000 *g* for 20 min. at room temperature and the supernatant treated at 4° with saturated $(NH_4)_2SO_4$ which had been neutralized with aq. NH_3 soln. The fraction obtained between 34 and 43% saturation from 45 g. of dried-pea powder was dissolved in 12 ml. of water and dialysed with rocking for 4·5 hr. at 4° against water. Fractionation at low temperatures with acetone or ethanol yielded less active enzyme preparations.

The clear, straw-coloured enzyme preparation could be stored at −15° for 2 weeks with 10% loss of activity and with 40% loss after 6 weeks. Heating for 10 min. at 50° and 55° resulted in 32 and 95% loss respectively of inosine 5′-phosphate-dehydrogenase activity.

Measurement of enzyme activity. The reaction mixtures were maintained at 25°. For the assay of inosine 5′ phosphate-dehydrogenase activity the following components were mixed: 0·2μmole (approx.) of inosine 5′-phosphate, 0·4μmole (approx.) of DPN, 100μmoles of KCl,

80μmoles of 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, pH 8·0, 0·2 ml. of enzyme (containing approx. 40–50μg. of protein); total volume, 3·0 ml. In some early experiments 5μmoles of GSH were also added. The time of incubation and enzyme concentration were adjusted so that not more than 50% conversion of inosine 5′-phosphate occurred during the reaction period. Under these conditions the reaction rate remained approximately constant.

The oxidation of inosine 5′-phosphate was followed by observing the increase in extinction at 340 mμ due to the formation of reduced diphosphopyridine nucleotide (DPNH). Measurements were carried out in a Beckman model DU spectrophotometer with 1 cm. cuvettes. Two series of control reaction mixtures, (i) omitting inosine 5′-phosphate and (ii) omitting DPN, were always prepared. There was usually a small increase in $E_{340\ m\mu}$ in (i) and a barely significant change in (ii). The results are expressed as the increase in $E_{340\ m\mu}$ of the complete reaction mixtures after allowing for any change in the controls. In experiments where DPN was replaced by TPN, the reaction was also followed at 340 mμ. Determination of the increase in extinction at 290 mμ (Magasanik *et al.* 1957) due to the formation of xanthosine 5′-phosphate was unsatisfactory because of the high extinction of the enzyme preparation at this wavelength.

Chromatography. The nucleotides were separated on paper chromatograms, detected and eluted as described previously (Turner & Turner, 1958; Kirkland & Turner, 1959). Multiple applications of 24μl. portions were made on each spot.

Determination of protein. The protein concentration of the enzyme preparations was estimated by the spectrophotometric method of Warburg & Christian (1941).

RESULTS

Inosine 5′-phosphate-dehydrogenase activity

When reaction mixtures of the composition described for the assay of inosine 5′-phosphate-dehydrogenase activity were incubated, an increase in extinction at 340 mμ, which was attributed to DPNH formation, was observed.

Xanthosine 5′-phosphate was isolated from a reaction mixture of the composition described for the assay of inosine 5′-phosphate dehydrogenase. The reaction was allowed to proceed for 120 min. and stopped by heating at 95° for 5 min. After centrifuging, the supernatant was applied to Whatman no. 3MM papers which were developed for 16 hr. with the ethanol–M-ammonium acetate, pH 7·5 (5:2, v/v), solvent of Paladini & Leloir (1952). The areas corresponding to a spot moving slightly slower than inosine 5′-phosphate were cut out, eluted with water and the extract was evaporated to dryness at 50° in a stream of N_2. Two drops of water were added and the extract was applied to a Whatman no. 3MM paper, which was developed for 18 hr. with *iso*butyric acid–aq. NH_3 soln. (sp.gr. 0·880)–water (66:1:33, by vol.) as solvent (Pabst Laboratories, 1956). A spot which travelled a little more slowly than inosine 5′-phosphate was eluted with 3 ml. of 50 mM-phosphate (KH_2PO_4–Na_2HPO_4) buffer, pH 7 (Kirkland & Turner, 1959). The absorption spectrum was very similar to that of xanthosine. Maxima were obtained at 248 and 276 mμ with a minimum at 267 mμ. Xanthosine 5′-phosphate prepared by the deamination of

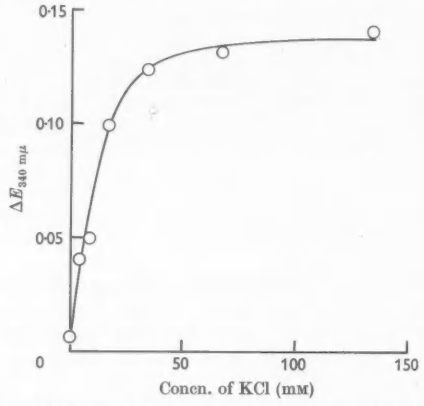

Fig. 1. Effect of KCl concentration on inosine 5′-phosphate-dehydrogenase activity. Reaction mixtures contained 0·26μmole of inosine 5′-phosphate, 0·44μmole of DPN, 80μmoles of tris–HCl buffer, pH 8·0, 0·2 ml. of enzyme (containing 48μg. of protein); total volume, 3·0 ml. Concentration of KCl was varied as shown. Time of incubation, 30 min. Temp. 25°.

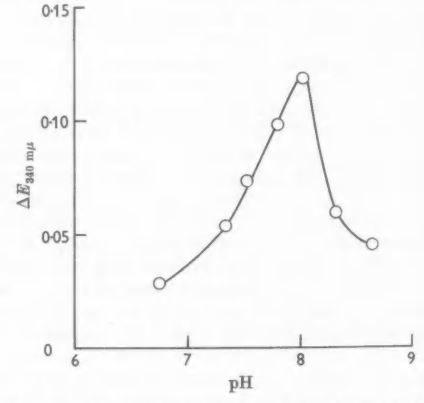

Fig. 2. Effect of pH on inosine 5′-phosphate-dehydrogenase activity. Reaction mixtures contained 0·22μmole of inosine 5′-phosphate, 0·45μmole of DPN, 100μmoles of KCl, 80μmoles of tris–HCl buffer of required pH, 0·2 ml. of enzyme (containing 42μg. of protein); total volume, 3·0 ml. Time of incubation, 15 min. Temp. 25°.

guanosine 5′-phosphate by nitrite (Abrams & Bentley, 1959) gave maxima at 248 and 276 mμ and a minimum at 267 mμ. Magasanik *et al.* (1957) found that xanthosine 5′-phosphate formed by the oxidation of inosine 5′-phosphate by the enzyme from *A. aerogenes* had maxima at 248 and 277 mμ with a minimum at 265 mμ at pH 7.

In some preliminary experiments a slight stimulation of DPNH formation was obtained on the addition of GSH. Subsequent experiments failed to confirm those observations and GSH was therefore omitted from the reaction mixtures.

Cofactor requirements. The enzyme was largely inactive in the absence of a suitable univalent cation. Fig. 1 shows the effect of K^+ ion concentration on inosine 5′-phosphate-dehydrogenase activity. The reaction rate reached a maximum at a concentration of between 33 and 66 mM-KCl; further increase in KCl concentration to 0·13 M did not affect the rate of reaction.

When KCl (final concn. 33 mM) in the reaction mixture was replaced by the same concentrations of NH_4Cl or NaCl the rates of reaction were 47 and 6% respectively of those obtained with KCl. The addition of NaCl (33 mM) to a reaction mixture containing KCl (33 mM) had no effect.

Effect of pH. The activity of the enzyme was studied in a series of buffers containing tris (80 μmoles) and HCl over the range pH 6·7–8·6 (Fig. 2). The pH–activity curve showed a sharp optimum at approximately pH 8·0.

Effect of concentration of substrates. The effect of increasing DPN concentration on the rate of reaction is shown in Fig. 3. The optimum concentration was greater than 0·6 mM and from a Lineweaver & Burk (1934) plot the K_m for DPN was calculated to be approximately 0·25 mM.

Variation in inosine 5′-phosphate concentration also affected the reaction velocity (Fig. 4); a Lineweaver–Burk plot gave K_m approximately 0·026 mM for inosine 5′-phosphate.

Effect of inhibitors on inosine 5′-phosphate-dehydrogenase activity. The effects of several inhibitors on the rate of reaction are shown in Table 1. Mercuric chloride, *p*-hydroxymercuribenzoate and iodoacetate strongly inhibited inosine 5′-phosphate-dehydrogenase activity. Phosphate, arsenate and fluoride were without effect.

Specificity of the pea-seed inosine 5′-phosphate dehydrogenase. Inosine could replace inosine 5′-phosphate to a small extent as substrate for the

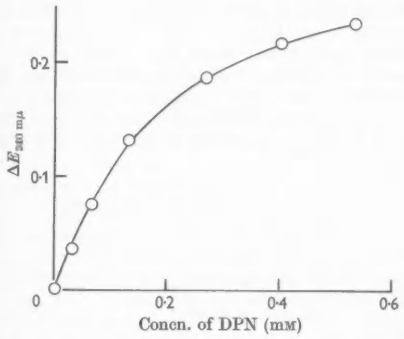

Fig. 3. Effect of DPN concentration on inosine 5′-phosphate-dehydrogenase activity. Reaction mixtures contained 0·26 μmole of inosine 5′-phosphate, 100 μmoles of KCl, 80 μmoles of tris–HCl buffer, pH 8·0, 0·2 ml. of enzyme (containing 48 μg. of protein); total volume, 3·0 ml. Concentration of DPN was varied as shown. Time of incubation, 30 min. Temp. 25°.

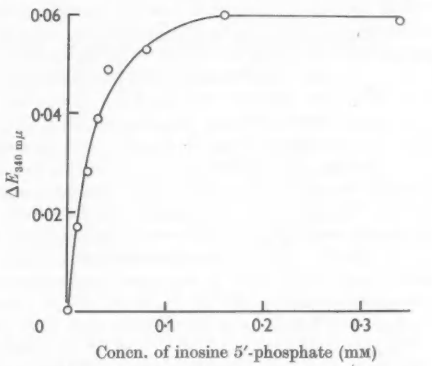

Fig. 4. Effect of inosine 5′-phosphate concentration on inosine 5′-phosphate-dehydrogenase activity. Reaction mixtures contained 0·41 μmole of DPN, 100 μmoles of KCl, 80 μmoles of tris–HCl buffer, pH 8·0, 0·1 ml. of enzyme (containing 22 μg. of protein); total volume, 3·0 ml. Concentration of inosine 5′-phosphate was varied as shown. Time of incubation, 30 min. Temp. 25°.

Table 1. *Effect of inhibitors on inosine 5′-phosphate-dehydrogenase activity*

Reaction mixtures contained 0·22 μmole of inosine 5′-phosphate, 0·44 μmole of DPN, 100 μmoles of KCl, 80 μmoles of tris–HCl buffer, pH 8·0, 0·2 ml. of enzyme (containing 42 μg. of protein), total volume, 3·0 ml. Inhibitors were added as shown. Time of incubation, 30 min. Temp. 25°.

Inhibitor	Final concn. (mM)	Inhibition (%)
Inorganic phosphate	10	0
Arsenate	10	0
Fluoride	10	0
$HgCl_2$	0·5	100
p-Hydroxymercuribenzoate	1	97
Iodoacetate	10	98
Iodoacetate	5	85
Iodoacetate	1	25

enzyme. When inosine 5′-phosphate (0·2μmole) was replaced by inosine of the same concentration in reaction mixtures of the composition described for the assay of inosine 5′-phosphate dehydrogenase the rate of reduction of DPN was 8% of that given with inosine 5′-phosphate as substrate.

Inosine 5′-phosphate dehydrogenase was essentially specific for DPN as substrate. When TPN (0·4μmole) was substituted for DPN (0·4μmole) in reaction mixtures of the composition used for the assay of inosine 5′-phosphate dehydrogenase, the rate of reaction was only 2% of that given by DPN; this may have been due to the presence of DPN as impurity in the TPN preparation.

DISCUSSION

The present investigation has shown that active preparations of inosine 5′-phosphate dehydrogenase may be readily obtained from pea seeds.

The inosine 5′-phosphate dehydrogenase from pea seeds has similar properties to the enzyme from *Aerobacter aerogenes* (Magasanik *et al.* 1957). The pH–activity curves for the two enzymes are very similar, the optimum pH for the pea-seed enzyme being pH 8·0 and for the bacterial enzyme pH 8·1. The inosine 5′-phosphate dehydrogenase of pea seeds required a suitable univalent cation for activity; K^+ ions were the most effective of those tested and NH_4^+ ions in the same concentration gave approximately half the rate given by K^+ ions. Sodium ions had only a slight effect and were not inhibitory in the presence of the optimum concentration of K^+ ions. Magasanik *et al.* (1957) found that K^+ or NH_4^+ ions were required for activity of the inosine 5′-phosphate dehydrogenase from *A. aerogenes*. These authors found that the latter enzyme was not active with either inosine or TPN as substrates: with the pea-seed enzyme inosine and TPN gave 8 and 2% respectively of the rates obtained with inosine 5′-phosphate and DPN as substrates.

The strong inhibition of pea-seed inosine 5′-phosphate dehydrogenase by *p*-hydroxymercuribenzoate and iodoacetate indicated that the enzyme possessed sulphydryl groups essential for activity. Although Magasanik *et al.* (1957) did not report inhibition by sulphydryl reagents they found that glutathione and cysteine stimulated the activity of the inosine 5′-phosphate dehydrogenase from *A. aerogenes*.

The role of inosine 5′-phosphate dehydrogenase in bacteria in the formation of nucleic acid guanine was indicated by the work of Magasanik *et al.* (1957) and Moyed & Magasanik (1957) with purineless mutants, and the enzyme may perform the same function in plants in the formation of guanine derivatives. Guanosine triphosphate has been shown to participate in several reactions, including the formation of succinyl-coenzyme A (Sanadi, Gibson & Ayengar, 1954; Sanadi, Gibson, Ayengar & Jacob, 1956), the oxaloacetate-carboxylase reaction (Kurahashi, Pennington & Utter, 1957) and the synthesis of adenylosuccinate (Lieberman, 1956). Other derivatives such as guanosine diphosphomannose and guanosine diphosphofucose may be of significance in the metabolism of those plants which accumulate polysaccharides containing mannose and fucose.

SUMMARY

1. Inosine 5′-phosphate dehydrogenase, which catalyses the reaction

$$\text{inosine 5′-phosphate} + \text{diphosphopyridine nucleotide} \downarrow \text{xanthosine 5′-phosphate} + \text{reduced diphosphopyridine nucleotide},$$

was found in pea-seed extracts.

2. Potassium ions were necessary for the reaction; NH_4^+ and, to a slight extent, Na^+ ions could partly replace K^+ ions as cofactor for the enzyme.

3. The effects of K^+ ion concentration, pH and concentration of substrates on inosine 5′-phosphate-dehydrogenase activity were studied.

4. The enzyme was strongly inhibited by sulphydryl reagents.

5. The rate of reaction was very small when inosine 5′-phosphate was replaced by inosine or when diphosphopyridine nucleotide was replaced by triphosphopyridine nucleotide.

The work described in this paper was carried out as part of the joint-research programme of the Division of Food Preservation, C.S.I.R.O., and of the Botany School, University of Sydney. The authors wish to express their indebtedness to Dr J. R. Vickery, Chief, Division of Food Preservation, and Professor R. L. Crocker, Botany School, University of Sydney, in whose laboratories the work was carried out.

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Biochem. J. (1961) **79**, 151

Cytochrome Pigments from the Green Photosynthetic Bacterium *Chlorobium thiosulphatophilum*

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The early notion that the cytochrome pigments of living tissues are associated closely with the reduction of molecular oxygen has had to be modified because of two main findings. Hill & Scarisbrick (1951) demonstrated that green plants contain cytochrome *f*, a pigment specifically associated with photosynthetic tissues and not found in other parts of the plant. Postgate (1954) showed that the strictly anaerobic bacterium *Desulphovibrio desulphuricans* is rich in cytochrome c_3.

Elsden, Kamen & Vernon (1953) isolated cytochrome from the purple non-sulphur bacterium *Rhodospirillum rubrum*, which is both photosynthetic and predominantly anaerobic in its mode of life. Vernon & Kamen (1954) subsequently demonstrated the presence of cytochrome pigments in extracts of the strictly anaerobic photosynthetic sulphur bacteria *Chromatium* and *Chlorobium*. In these organisms the simplest type of photosynthetic cytochrome system might be expected, uncomplicated by any possibility of oxygen metabolism, and it therefore appeared to be of interest to examine the cytochromes in these organisms more closely. The pigment from *Chromatium* has been prepared, and its properties investigated, by Newton & Kamen (1956).

The present paper describes the isolation and properties of two cytochromes, resembling each other in many of their properties, from the green sulphur bacterium *Chlorobium thiosulphatophilum*. A preliminary account of part of this work has appeared (Gibson & Larsen, 1955).

METHODS

Organisms and culture methods. A strain of *Chlorobium thiosulphatophilum* kindly supplied by Dr J. Lascelles of Oxford was used for the main part of the work. Small-scale experiments with a second strain, obtained from Dr C. B. van Niel, showed that the two component pigments were again present.

The maintenance and culture of the organisms has been fully described by Larsen (1952). For large-scale growth Larsen's medium, modified so as to contain 0·2% of $Na_2S_2O_3$ and 0·4% of $NaHCO_3$ (both w/w), was used to fill completely 25 l. carboys, whose necks were sealed with rubber bungs. Five vessels were illuminated with 12 60 w incandescent bulbs, and the circulation of air round them was limited so as to produce a temperature of about 25° in the medium. The vessels were filled the day before they were inoculated with 200 ml. of a 2-day culture of the organisms grown in a medium containing 0·1% of $Na_2S_2O_3$ and 0·2% of $NaHCO_3$ (w/w). Growth was complete in 4–5 days, when the cells were harvested with a Sharples supercentrifuge. The yield ranged from 40 to 60 g. wet wt. equivalent to 14–20 g. of dry matter. The bacterial paste was stored as such at −20°, or spread thinly on glass plates and dried *in vacuo* over H_2SO_4 at room temperature or freeze-dried. The frozen cells and the dried preparations were both stable over a period of many months.

Phosphate buffers. These were prepared from KH_2PO_4 solutions adjusted to the required pH with NaOH, a Pye pH meter being used.

Estimation of cytochrome concentration. A hand spectroscope was used to follow roughly the distribution of cytochrome during purification. A quantitative estimate of the cytochrome content of the different fractions was obtained by measuring the extinction difference between the fully oxidized and reduced preparations at the absorption peaks of the α and Soret bands. A suitable dilution was made in 0·05 M-phosphate buffer, pH 7·0, and approx. 5 mg. of solid sodium dithionite added to 3 ml. in the spectrophotometer cuvette. The extinction of the solution at 417 and 554 mμ was measured with a Unicam SP. 500 or SP. 600 spectrophotometer with cells of 1 cm. light-path. A second portion was diluted to the same extent in a solution containing the same phosphate concentration and $K_3Fe(CN)_6$ to a final concentration of 0·02 M. In this case the blank cell of the spectrophotometer contained the same concentration of $K_3Fe(CN)_6$, and extinction measurements were made at the same wavelengths. In practice it was found that the pigments were entirely in the oxidized state after the first two steps of the purification had been carried out, and it was then possible to measure the extinction difference in the same solution, before and after the addition of a trace of dithionite. The value of the estimates of cytochrome content of the bacterial extracts themselves is uncertain, since such extracts are highly coloured, and it could be seen that there was a slight bleaching of these pigments on addition of dithionite. Cytochrome content of fractions was calculated in the latter preparations by appropriate factors obtained from a highly purified sample of cytochrome 554 whose extinctions and dry weight had been determined.

Absorption spectra. The positions of the absorption bands of the cytochromes and their derivatives were found with a Hartridge reversion spectroscope. Absorption spectra were obtained with a Unicam SP. 500 spectrophotometer, and with an Optica model CF4 recording spectrophotometer.

Protein estimation and determination of purity of cytochrome. A measure of total protein in extracts and fractions was obtained by measuring the extinction of suitably diluted samples at 280 mμ. Purification of the cytochromes was followed by using the ratio of extinction (E) at 280 mμ to that at the Soret maximum in the reduced form.

Haemin estimation. Keilin & Hartree's (1951) method was used, modified as follows so as to use less material. The solution to be analysed was added to 0·1 ml. of N-NaOH contained in a 5 ml. graduated cylinder. Pyridine (0·4 ml.), followed by a trace of dithionite, was added, and the whole made up to 2 ml. with water and mixed. The pyridine haemochromogen was measured after exactly 30 min. in a Hilger Spekker absorptiometer in 1 cm. microcells and with a 605 filter. A standard curve was prepared with crystalline haemin.

Iron estimation. The iron content of purified cytochrome solutions was determined by a modification of Coombs's (1936) method for the determination of total iron in blood.

Pyrex incineration tubes (4 in. by $\frac{5}{16}$ in. internal diam.) were calibrated at 2 ml. The samples for estimation were pipetted into the tubes and, if of greater volume than 0·2 ml., were evaporated to about 0·2 ml. Hydrogen peroxide (1 drop of 100 vol.) was added, and the solution evaporated to dryness. If charring occurred, a further drop of peroxide was added and the evaporation repeated. Glass-distilled water (approx. 0·5 ml.) was added and evaporated; this step was repeated once more. The following solutions were then added in the order given and mixed: glass-distilled water (1·0 ml.); 40% (w/v) sodium acetate 0·5 ml.; 20% (w/v) glucose 0·2 ml.; αα′-dipyridyl solution 0·1 ml.

The αα′-dipyridyl solution was prepared by dissolving 0·117 g. of the solid in 1·5 ml. of N-HCl, and diluting the solution to 25 ml. with glass-distilled water. The incineration tubes were placed in a boiling-water bath for 10 min., allowed to cool and the contents made up to the mark. The intensity of the red colour was measured in the Hilger Spekker absorptiometer, in microcells of 1 cm. light-path and with 604 filters. A standard curve was prepared by using ferrous ammonium sulphate.

Hydrogen peroxide was found to be a more suitable oxidant than HNO_3, since it did not lead to the formation of a crystalline precipitate when dried whole bacteria were used for estimation of iron. The step of evaporating the incinerated material repeatedly from water prevented the subsequent formation of a yellow colour which was presumably due to oxidation of glucose by residual peroxide.

Measurement of oxidation–reduction potential. The potential of both cytochromes was found to lie between the ranges covered by the ferri- –ferro-cyanide and the ferri- -ferro-oxalate couples. Measurements of potential were therefore made by titration of the reduced cytochromes with ferricyanide, the change in potential being measured directly against a standard calomel electrode.

The cytochrome solutions (5 ml., extinction at 554 mμ approx. 0·5 in 0·1 M-phosphate buffer, pH 7·0) were placed in small weighing bottles, of about 10 ml. capacity. A fine stream of O_2-free N_2 was passed into the solution through a fine capillary to make and maintain anaerobic conditions. A small platinum electrode was placed in the solution and connected to a Pye pH meter used as a potentiometer. The meter was also connected to a standard calomel electrode immersed in a saturated solution of KCl, and the circuit was completed by means of an agar bridge. Potassium ferricyanide (0·1 M) was added in 1 μl. amounts from an Agla syringe (Burroughs Wellcome and Co., London) and the titration curve plotted. The solution was viewed through a hand spectroscope to confirm the range in which the cytochrome was being oxidized, and the E'_0 obtained from the centre of the plateau in the titration curve.

RESULTS

Influence of growth conditions

Cytochromes were detected in all cultures examined, but the yield appeared to be somewhat greater from cultures that had completed their growth. Cultures in which the colour of the cells varied from a deep green to almost yellow also appeared to contain roughly equal amounts of cytochrome.

Extraction of cytochromes

Acetone-dried powders. The cytochrome bands were first recognized in suspensions of acetone-dried powders to which a trace of dithionite had been added. The cytochromes could be brought partially into solution by extraction with buffers of

pH ranging from 6·0 to 9·0, but the bands were still strong in the residual cellular material.

Dried cells. Extracts prepared from cells which had been dried *in vacuo* at room temperature or from the frozen state all contained cytochromes. At pH 9 the extracts were deep green, and had an absorption spectrum resembling that of fresh cells, with strong bands at 745 and 810 mμ. At lower pH (6·0–7·5) extracts were brownish in colour and showed in addition two sharp bands at 610 and 560 mμ, which were probably due to porphyrin. The yield of cytochromes appeared to be a little higher at high pH, but the accuracy of the estimations in crude extracts is somewhat suspect.

Wet cells. The cells of *Chlorobium* are relatively fragile and cytochromes could be extracted from freshly harvested cells by a variety of techniques, such as incubation with solutions of cetyltrimethylammonium bromide, grinding the cells with sand or powdered glass, breaking the cells in the Hughes press, or sonically with a 1 kw Mullard oscillator (Mullard Ltd., London) or even by freezing and thawing. This last technique was generally used both because of its simplicity and its comparative gentleness which, it was thought, was unlikely to cause denaturative changes in the pigments. The influence of pH on extraction was the same as when dried cells were used, and extraction at pH 9·2 in 5% (w/v) sodium borate was adopted as routine procedure.

Separation of cytochrome components

Batches of about 50 g. dry wt. of cells were used for large-scale preparation of cytochromes. A typical preparation is illustrated in Table 1.

Extraction. The cells were suspended in five times their (dry) weight of 5% (w/v) sodium borate solution, and frozen at −20° and thawed at +30°; the freezing and thawing were repeated once. The suspension was then centrifuged at 20 000*g* for 15 min. and the supernatant collected. The cell paste was washed twice with 2 vol. of borate, resuspended in a similar volume and frozen and thawed again, and centrifuged and washed once. Further washing yielded little more cytochromes, and the cell residue was discarded.

First ammonium sulphate precipitation. The dark-green extract was treated with 20 g. of solid ammonium sulphate/100 ml.; the heavy green precipitate was removed by centrifuging or filtration and discarded. The light-brown filtrate was dialysed against running tap water for 48 hr., or until a small sticky precipitate, which was discarded, was formed.

Separation of cytochromes 553 and 554 with cation-exchange resin. Treatment of the dialysed brown solution with the ammonia form of Amberlite IRC50 (Margoliash, 1954) brought about the adsorption of part of the cytochrome. The pigment remaining in solution was not taken up even if

Table 1. *Separation and purification of cytochromes from borate-extracted cells*

	Total volume	E_{280}*	E_{417}* ox.	E_{417}* red.	E_{554}* ox.	E_{554}* red.	Total cytochrome† (mg.) From Δ 417	Total cytochrome† (mg.) From Δ 554	$E_{280}/E_{417\ red.}$	Recovery‡ (%)
(1) Borate extract	500	56	31	32·6	3·51	3·7	352	148	14·9	100
(2) 20% (w/v) $(NH_4)_2SO_4$ supernatant	485	37·2	3·84	4·11	0·4	0·56	57·7	121	9·1	82
(3) (2), dialysed	735	16·8	2·01	2·31	0·198	0·312	98·2	131	7·3	89
				Cytochrome 553						
(4) Amberlite column effluent	720	16·2	1·62	1·77	0·294	0·342	47·5	54	9·2	36·5
(5) $(NH_4)_2SO_4$ ppt., dialysed	37·5	148	13·8	17·1	1·38	2·22	56	57	8·7	32·4
(6) Butan-1-ol subnatant, dialysed	44	47·7	6·18	7·89	1·62	2·1	33·2	33	6·05	22
				Cytochrome 554						
On Amberlite column, by difference	—	—	—	—	—	—	45·5	77	—	52·5
Ammonia eluate	6·2	16·5	48·5	65	3·9	9·8	45	57	0·254	39

* Dilutions were made in 1 mM-phosphate buffer of pH 7·0 so that the extinctions measured lay between 0·05 and 1·0 with cells of 1 cm. optical depth. Numbers given represent the extinctions of the undiluted solutions.

† Calculated from the values ($E_{red.-ox.}$ 0·1 ≡ 44 μg. of cytochrome at 417 mμ; $E_{red.-ox.}$ 0·1 ≡ 156 μg. of cytochrome at 554 mμ) obtained from a sample of cytochrome 554, $E_{280}/E_{417\ red.}$ 0·21, whose dry weight had been determined after freeze-drying

‡ Based on extinction differences at 554 mμ: borate-extract value was taken as 100%.

further portions of resin were added, and this treatment was therefore used to separate the basic cytochrome 554 from the acidic cytochrome 553. Amberlite was added to the solution (0·5 g./100 ml.) and the suspension stirred for 24 hr. at 4°. The resin was then pale pink. The solution was run through an Amberlite column (10 cm. × 1 cm.) to remove any residual traces of basic cytochrome. The resin from the batch treatment was added to the top of the column, and the whole washed with 20 ml. of water. The washing was added to the main bulk of the Amberlite effluent.

Further purification of cytochrome 553

Second ammonium sulphate treatment. The effluent from the Amberlite column still contained about half the total cytochrome, as measured by the difference in extinction at 554 mμ between the oxidized and reduced forms. Further ammonium sulphate fractionation did not effect substantial purification, and the effluent was therefore concentrated by total precipitation in saturated ammonium sulphate solution. The precipitate was collected by centrifuging, and the slurry obtained by stirring the sediment with a glass rod was transferred to a dialysis sac and dialysed against running tap water. In one preparation this dialysis was dispensed with, but the losses in the next step appeared in consequence to be greater than usual.

Butan-1-ol treatment. On shaking the dialysed solution from the previous step with 0·2 vol. of butan-1-ol in the cold a heavy precipitate was formed. Three layers separated on centrifuging: a small top layer of deep-green solvent, separated from a cloudy brownish pink aqueous layer by a thick layer of denatured protein. The subnatant was collected and the top layers were washed twice with 10 ml. of 0·01 M-phosphate buffer, pH 7·2. The combined subnatants were dialysed against running tap water until the smell of solvent had gone. Losses of cytochrome at this stage were variable and usually of the order of 50 % in spite of care being taken to carry out the step as rapidly as possible and in the cold. None the less it represented the best treatment devised, and fractionation with other solvents, such as ethanol and acetone, resulted in as great if not greater losses, and a smaller degree of purification.

Fractionation on columns of diethylaminoethylcellulose. The solution obtained from the last step was brownish pink, and contained other coloured material as well as cytochrome. The ratio of extinction at 280 mμ to that at the height of the Soret band (416 mμ) in the reduced form varied from 3·6 to 7. Columns of diethylaminoethylcellulose, prepared as described by Sober & Peterson (1956), were used to effect further purification. The phosphate concentration of the buffer flowing through the column had to be raised from 2 mM to 0·5 M at pH 7, or to 0·15 M at pH 6·5, before much movement of the cytochrome took place; in most runs, the cytochrome solution (2 ml. containing 15–20 mg. of protein) was run into the column with 10 ml. of buffer (2 mM), pH 7, the column was washed with two 50 ml. portions of buffer at 0·02 M and 0·2 M, pH 7, and then developed with 0·5 M-buffer, again at pH 7. When good separation of the cytochrome band from the green and brownish bands which preceded it had been obtained, the columns were extruded, cut and the sections eluted with M-phosphate buffer, pH 7. About 50 % of the pigment was usually recovered in the best fractions; both leading and tailing fractions of the main band were contaminated with other material absorbing at 280 mμ. The use of calcium phosphate-gel columns (Swingle & Tiselius, 1951) supported either on Celite or cellulose was investigated, but although considerable purification was achieved much of the pigment became permanently adsorbed and could not be eluted even

Table 2. *Further purification of cytochrome components*

Best values are given.

	Cytochrome 554		Cytochrome 553	
Treatment	$E_{280}/E_{417\ red.}$	$E_{417}/E_{554\ red.}$	$E_{280}/E_{416\ red.}$	$E_{416}/E_{553\ red.}$
Amberlite: two treatments	0·25	6·44	—	—
	0·24	7·16	—	—
Butan-1-ol	—	—	6·4	4·2
	—	—	3·6	3·3
Calcium phosphate-gel columns	0·234	6·26	1·83	5·98
	—	—	1·71	4·84
	—	—	1·88	6·55
Zone electrophoresis	0·22	6·76	1·18	5·3
	0·171	7·27	—	—
Carboxymethylcellulose	0·199	7·02	—	—
	0·18	6·8	—	—
Diethylaminoethylcellulose	—	—	0·74	5·22
	—	—	0·75	5·5

with M-phosphate. Zone electrophoresis in a cellulose column (Flodin & Porath, 1954) likewise brought about approximately fourfold purification in the best fractions, but the total recoveries were in all cases below 50%. Results are summarized in Table 2.

Further purification of cytochrome 554

The Amberlite column on which the basic cytochrome had been collected was washed with a further 200 ml. of water and the pigment eluted with 1% (v/v) ammonia (sp.gr. 0·880). The sharp band of cytochrome was collected in a small tube and the ammonia removed *in vacuo* over sulphuric acid.

The ratio E_{280}/E_{417} for the reduced form of the cytochrome at this stage was usually about 0·26, and repetition of the Amberlite treatment as described by Margoliash (1954) for cytochrome *c* gave products in which this ratio was never lower than 0·24. A sample of this degree of purity run in the ultracentrifuge showed the presence of about 25% of a colourless component, which separated from the lighter-coloured material remaining at the top of the cell.

Further purification was effected on columns of carboxymethylcellulose prepared from 2 g. of the powder suspended in 2 mM-phosphate buffer as described by Sober & Peterson (1956). The pigment solution (2 ml., containing not more than 15 mg.) was washed into the column with 20 ml. of the same buffer. The column was then washed successively with 50 ml. portions of 5 mM-, 0·02 and 0·05M-phosphate buffer, and the cytochrome was finally brought off with 0·1M-buffer, all at pH 7. The red cytochrome band, which showed some tendency to separate into a main, leading band of the scarlet oxidized pigment, and a tailing cherry-red reduced portion, was both preceded and followed by some colourless protein, as judged by the $E_{280}/E_{417\ red.}$ ratio. In the best runs, 65–75% of the pigment was recovered in the purest fractions. Other purification procedures were investigated, but produced poorer results. Chromatography on a column of calcium phosphate gel (Swingle & Tiselius, 1951) supported on Celite or cellulose powder produced little decrease in the ratio E_{280}/E_{417}, and recovery of the pigment was poor. Zone electrophoresis in a cellulose column (Flodin & Porath, 1954) in one instance gave a fraction in which the ratio was as low as 0·171, but results were very variable, and this could not be repeated. Recoveries from the columns were very poor, as much as 70% of the pigment remaining on the cellulose. Results are summarized in Table 2.

Properties of the cytochromes

Stability. The cytochromes from *Chlorobium* resemble cytochrome *c* in being extremely resistant to denaturation. They are stable in the range pH 3·5–10·0, and can also be placed in a boiling-water bath for 5 min. without permanent changes being caused; the absorption bands of the reduced compounds disappear on heating but are restored when the solution cools.

Reaction with other compounds. Neither of the isolated cytochrome pigments reacts with carbon monoxide or cyanide. The absence of reaction with carbon monoxide has been used as a test for denaturation during purification. Compounds are formed between nitric oxide and the oxidized compounds. While in the crude state the pigments are somewhat auto-oxidizable; this property disappears on purification and the purest preparations were stable in the reduced form in air.

Isoelectric points. These have not been determined accurately, but from the method of preparation they are known to be widely different for the two pigments. Cytochrome 554 is rather more firmly held on Amberlite than is cytochrome *c*, and its isoelectric point is therefore probably rather higher than that of cytochrome *c* (10). The isoelectric point of cytochrome 553 lies well to the acidic side of neutrality, judging by its behaviour on carboxymethylcellulose.

Absorption bands. The positions of the centres of the absorption bands of both components in the reduced and oxidized states and of the nitric oxide compounds are given in Table 3, together with those of heart-muscle cytochrome *c* for comparison. In Fig. 1, the α- and β-bands of the purest preparations are shown, as measured in the recording spectrophotometer at a resolution of 1 Å. The sample of cytochrome 554 had $E_{280}/E_{417\ red.}$ 0·18, and was electrophoretically homogeneous; the

Table 3. *Wavelengths of absorption bands of cytochrome 553 and 554, with those of cytochrome c for comparison*

Form	Wavelengths (mμ)								
	Cytochrome 554			Cytochrome 553			Cytochrome *c*		
Oxidized	412	535	—	408	—	533	407	530	—
Reduced	417	523	554	416	522	553	415	520	550
Nitric oxide compound	414	530	570	413	530	568	411	—	565

cytochrome 553 ($E_{280}/E_{416\ red.}$ 1·06) contained about 27% of colourless impurity, as estimated by ultracentrifuging. A pure sample of cytochrome *c* is included for comparison. The Soret bands of all these preparations were symmetrical.

Nature of the prosthetic group. Paul's (1950) procedure was applied to solutions (1 ml.; E_{417} about 1) of the two components in order to determine the nature of the prosthetic group, a solution of cytochrome *c* being used for comparison. The cytochrome 554 solution required rather longer heating in the silver sulphate–acetic acid mixture in order to obtain good extinction of haemin than did the others. The absorption maxima in the blue were at 390 mμ for both cytochromes whereas that for cytochrome *c* haemin was at 388 mμ. In all probability therefore the prosthetic groups of the *Chlorobium* cytochromes are identical with that of heart-muscle cytochrome *c*.

Molecular size and homogeneity. An impure preparation of cytochrome 554 with $E_{280}/E_{417\ red.}$ 0·22 was run in the ultracentrifuge in 0·05M-phosphate buffer, pH 7·2. A colourless component was easily separated from the coloured material, which barely moved from the meniscus during 4 hr. at 58 500 rev./min. Such behaviour indicates that this cytochrome is a small molecule, probably of size similar to cytochrome *c*. Another sample, $E_{280}/E_{417\ red.}$ 0·18, was examined in the Kern electrophoresis apparatus (Kern A.G. Aarau, Switzerland) in 0·05M-phosphate buffer, pH 6·5 (potential gradient 6 v/cm., current 3·4 mA), and showed only one component.

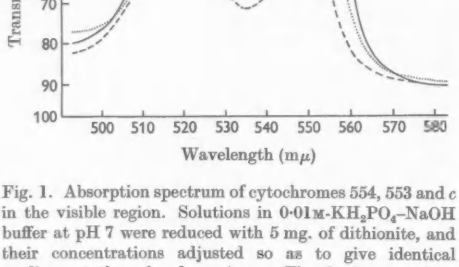

Fig. 1. Absorption spectrum of cytochromes 554, 553 and *c* in the visible region. Solutions in 0·01M-KH_2PO_4–NaOH buffer at pH 7 were reduced with 5 mg. of dithionite, and their concentrations adjusted so as to give identical readings at the α-band maximum. The Optica recording spectrophotometer at a resolution of 1 Å was used to draw the curves. - - -, Cytochrome *c* (iron content 0·43%); ..., cytochrome 553 ($E_{280}/E_{416\ red.}$ 1·06; 73% pure from ultracentrifuging); —, cytochrome 554 ($E_{280}/E_{417\ red.}$ 0·18; electrophoretically homogeneous).

A sample of cytochrome 553 ($E_{280}/E_{416\ red.}$ 1·06) was examined in the Kern electrophoresis apparatus and also in the ultracentrifuge. Both experiments showed the presence of about 27% of colourless protein. This impurity had a higher mobility than the coloured material in the electrophoresis experiment, which was carried out in 0·05M-phosphate buffer, pH 8·0, with a potential gradient of 6 v/cm. and a current of 3·4 mA. When the cytochrome 553, in 0·05M-phosphate buffer, pH 6·9, was ultracentrifuged at 59 780 rev./min. the impurity moved more slowly than the pigment. The main fraction, with which all the colour was associated, gave a value S_{20} 3·90 at a protein concentration of 3·5 mg./ml., and, assuming a partial specific volume of 0·735, it would appear that the molecular weight is considerably greater than that of the other cytochrome and probably of the order of 45 000–75 000.

Haemin and iron contents. The determination of haemin and iron contents was carried out in duplicate on a sample of cytochrome 554, $E_{280}/E_{417\ red.}$ 0·22, and gave mean values of 3·1 and 0·37% respectively. If these values are corrected for the impurity present, on the assumption that it contained neither iron nor haemin, and that the pure cytochrome has $E_{280}/E_{417\ red.}$ 0·18, these values become 3·79 and 0·452%, which agree closely with those for cytochrome *c*.

A sample of cytochrome 553 with $E_{280}/E_{416\ red.}$ 1·06 was first dialysed against three changes of distilled water, and its dry weight was obtained after freeze-drying. The dried material was then used for determining the iron content, and gave a value of 0·072% of iron. This preparation was not pure and if the value is corrected for the percentage purity estimated in the ultracentrifuge (73%), the iron content of the cytochrome is 0·113%.

Redox potential. The oxidation–reduction potentials of the two cytochromes as obtained by the titration method were: cytochrome 554, E'_0+140 mv; cytochrome 553, E'_0+163 mv. A sample of heart-muscle cytochrome *c* gave E'_0+262 mv.

Other haem compounds in Chlorobium. Elsden, Kamen & Vernon (1953) found that the photoheterotrophic organism *Rhodospirillum rubrum* contained in addition to cytochrome c_2 a cytochrome of the *b* type, with absorption bands at 560, 525 and 430 mμ. In the course of the present investigation many extracts of *Chlorobium* have been examined with the hand spectroscope, but in no case have any absorption bands appeared on reduction other than the fused bands of the cytochromes described above. Acetone-dried powders have been prepared from cells which had already been exhaustively extracted with borate to remove

cytochrome 554 and 553, and thick suspensions were prepared in 50% sucrose solution. In these preparations also no bands appeared on addition of dithionite other than faint ones at 554 and 523 mμ, due to traces of unextracted cytochromes of the known types. Vernon & Kamen (1954) have also described a haemoprotein of novel type (RHP) which is present in considerable quantities both in *Rhodospirillum* and *Chromatium*. This pigment should be easily recognizable in extracts since it reacts with carbon monoxide, and thus the sensitive test of a carbon monoxide difference spectrum can be applied. A number of preparations were made by citric acid extraction (Bartsch & Kamen, 1958) and by disruption in the Mullard sonic oscillator, and subsequently centrifuged in a Spinco preparative ultracentrifuge. The supernatant from the sonically disrupted cells was separated into two layers, an upper pale-green and a lower more deeply coloured one, and an acetone-dried powder was prepared from the almost black pellet at the bottom of the centrifuge tube. A faint indication of some material reacting with carbon monoxide to give a difference maximum at 424 mμ was obtained in suspensions of the acetone-extracted material suspended in 50% sucrose, but not in the other fractions, and in all the preparations examined, at most traces of carbon monoxide-combining material have been found. It must therefore be concluded that a RHP-like pigment is either absent from *Chlorobium* or present only in insignificant amounts.

DISCUSSION

The nomenclature of the cytochromes has become increasingly confused as more pigments have been described, with the result that pigments from widely differing sources have been consecutively numbered. According to the system suggested by Scarisbrick (1947) the full names of the two pigments described above would be *Chlorobium thiosulphatophilum* cytochrome 554 and *Chlorobium thiosulphatophilum* cytochrome 553. For the sake of brevity these have been referred to as cytochromes 554 and 553.

The two pigments resemble each other in many of their properties. The possession of the same prosthetic group would account for the closely similar position of the absorption bands, but Fig. 1 shows the considerable differences in fine structure of the purest preparations obtained which are also visible with the hand spectroscope. The α-band of cytochrome 554 is markedly asymmetrical, whereas those of cytochrome 553 and cytochrome *c* are less obviously so. In all three pigments the β-band is composite, and the relative intensities of the α- and β-bands are quite different for the three compounds. In addition, as shown in Table 2, the ratio of the intensities of absorption at the Soret maximum and at the α-band maximum is much higher for cytochrome 554 than for cytochrome 553, which has a ratio close to that for cytochrome *c*.

In other properties the pigments show well-marked differences. Whereas the protein of cytochrome 554 is basic in character, that of cytochrome 553 is acidic, and the molecules also differ considerably in size. The basic cytochrome 554 is small, and would appear from the ultracentrifuge run to be about the same size as cytochrome *c*. The iron content of the preparation indicates a minimum molecular weight of about 12 400. Similar calculations for cytochrome 553 indicate a molecular weight of about 50 000 based upon one haem iron per molecule or 45 000–75 000 on the basis of sedimentation rate. The molecule of cytochrome 553 is therefore among the largest cytochrome molecules reported, having a molecular weight of about half that of cytochrome *f* from green plants, which contains two haem groups per molecule (Davenport & Hill, 1952). Among photosynthetic bacteria, the cytochrome from *Chromatium* is assigned a molecular weight of about 30 000 (Newton & Kamen, 1956); the pigment from *Rhodospirillum rubrum*, cytochrome c_2, is about 13 000 (Kamen & Takeda, 1956). Both these pigments, like the *Chlorobium* cytochromes, appear to contain one haem group per molecule.

The two pigments may be inter-related in that one is derived from the other, but it is unlikely that such a conversion could be the result of the isolation procedures. Extractions of the pigments have been carried out by a wide range of techniques, both mild and rather drastic, and over a range pH 6·0–9·0, and in every case the two pigments were found to be present in approximately equal amounts. *Chlorobium* is not alone in possessing more than one cytochrome of similar absorption spectrum. Thus Olson & Chance (1958) have obtained evidence for the existence of a number of components in *Chromatium*, Trudinger (1958) has separated five cytochromes with α-band positions from 550 to 557 mμ from extracts of *Thiobacillus* and in animal tissues cytochrome c_1 resembles cytochrome *c* closely in its absorption spectrum (Keilin & Hartree, 1955).

The redox potentials of the *Chlorobium* cytochromes are lower than that of cytochrome *c*, but higher than the value of −10 mv given by Newton & Kamen (1956) for the pigment from *Chromatium*. All these cytochromes, and in addition that isolated by Postgate (1956) from *Desulphovibrio*, with the remarkably low E'_0 of −205 mv, and cytochromes c_2 from *Rhodospirillum* and *f* from green plants, with E'_0 at 340 and 360 mv respectively, have α-bands lying between 550 and 555 mμ and

appear to contain identical prosthetic groups. It has become increasingly clear over recent years that the group of *c*-type cytochromes is functionally a remarkably heterogeneous one, and that the possession of absorption bands in closely similar positions does not imply any close similarity in other properties.

The cytochromes of *Chlorobium* are present in considerable amounts in the cells, and up to 0·5% of the dry weight of the cells has been extracted as cytochrome, though the exact measurement of cytochrome content of extracts is difficult because of the presence of other pigments affected by reducing agents. Acetone-dried powders prepared from borate-extracted cells still showed the absorption bands at 554 and 523 mμ, and the total cytochrome content forms a substantial part of the cell. On the other hand, as far as could be ascertained, these two components are the only haemoproteins present in the cells in significant amounts.

It is now clear that the possession of cytochrome pigments peculiar to themselves is characteristic of photosynthetic tissues and organisms, and the work of Duysens (1953) and Olson & Chance (1958) has made it clear that changes in absorption on illumination correspond to changes in the state of oxidation of cytochrome-like pigments. These changes do not appear to be very rapid, and at what stage in the complex series of changes initiated by illumination the cytochrome functions remains obscure. It may be significant for the photosynthetic sulphur bacteria that Trudinger (1958) has isolated cytochromes from a *Thiobacillus*, and shown that one of these, with an α-band maximum at 553·5 mμ and E'_0 +210 mv, is reduced by an enzyme which can oxidize thiosulphate, either this cytochrome or ferricyanide being used as acceptor. Some indications have been obtained that the rate of reduction of 2:6-dichlorophenol-indophenol by thiosulphate in the presence of a crude preparation from *Chlorobium thiosulphatophilum* is increased when cytochrome 554 is added; a clarification of this effect must, however, await a separation and identification of the enzymic steps involved.

SUMMARY

1. Cytochrome pigments have been detected in extracts of *Chlorobium thiosulphatophilum* prepared by different techniques.

2. Two pigments have been separated with widely different isoelectric points.

3. The basic pigment, which has been obtained in a homogeneous state, has absorption maxima in the reduced form at 554, 523 and 417 mμ, is not auto-oxidizable and forms compounds with nitric oxide, when in the oxidized state, but not with cyanide or carbon monoxide when oxidized or reduced. The E'_0 value is 140 mv. The prosthetic group is closely related to that of cytochrome *c*. The molecular weight is about 12 000 and there is one haem group per molecule.

4. The acidic pigment, the best preparation of which still contained about 27% of colourless impurity, has absorption maxima in the reduced form at 553, 522 and 417 mμ, and resembles the basic pigment in general properties. The E'_0 value is 163 mv. The molecular weight is 45 000–75 000 from the ultracentrifuge study and the maximum molecular weight is about 50 000 from the haem content.

5. These pigments together account for up to 0·5% of the dry weight of the bacteria.

6. No other haem compounds have been detected in significant amounts.

My thanks are due to Mr Bowen of the Biochemistry Department, Leeds, and to Dr P. Johnson of the Department of Colloid Science, Cambridge, for the ultracentrifuge studies, and also to Dr T. Hofmann of the Department of Biochemistry in Sheffield, for carrying out the electrophoresis runs.

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Distribution of Fatty Acids in Lipids of Rat Brain, Brain Mitochondria and Microsomes

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The importance of lipids as structural elements of particulate enzyme systems is being increasingly realized (e.g. Green, 1959; Hokin & Hokin, 1959). The extent to which the lipids themselves participate in the metabolic function of the structures in which they are found and the specificity of the lipids for particular structures and functions are uncertain. The present study represents part of a search for lipids of specific structure in subcellular fractions of different organs. The underlying assumption is that the occurrence of a lipid with a particular structure in the corresponding cell organelle (or fraction thereof) of organs with different total lipid composition may give an indication of the metabolic function of the lipid.

Experimental data on the detailed composition of lipids in different tissues are scarce, and on the fatty acid distribution within lipids almost non-existent. In view of the variety of tissues used for research on subcellular organelles, and of the probable metabolic importance of lipids, a more detailed knowledge of tissue lipids is desirable.

The present study provides some new data for rat brain, brain mitochondria and microsomes. The study is based on partition of total lipid extracts by silicic acid chromatography and gas–liquid-chromatographic analysis of the fatty acid content of separated phospholipids.

EXPERIMENTAL

Materials

Rats. These were of the Wistar strain, approx. 300 g. in weight, fed on a standard diet of rat cubes supplied by Oxo Ltd. (London).

Chemicals. All solvents were of analytical grade, except ethanol, which was 'azeotropic'. Palmital dimethylacetal was kindly supplied by Dr Marjorie G. Macfarlane and Dr G. M. Gray.

Diazomethane was prepared from *N*-methyl-*N*-nitroso-toluene-*p*-sulphonamide (British Drug Houses Ltd., Poole, England). Choline chloride was recrystallized 'Laboratory reagent' (British Drug Houses Ltd.).

Analytical methods

Dry weights. Dry weights of tissue fractions were determined as 'acid-insoluble dry matter' (Werkheiser & Bartley, 1957). Dry weights of lipid extracts were determined after evaporation of the extracting solvent *in vacuo* at room temperature.

Phosphorus. This was determined by the method of Berenblum & Chain (1938), as modified by Bartley (1953). Wet ashing was by the method of Hanes & Isherwood (1949) as modified by Werkheiser & Bartley (1957).

Determination of acyl ester. The method used was a modification of that of Rapport & Alonzo (1955), giving linear standard curves with up to 4 μmoles of ester, but with variable colour yields.

The concentrations of NaOH and hydroxylamine were increased to 5%, w/v (2 g. of reagent dissolved in 2 ml. of water and diluted with 38 ml. of ethanol). Esters were dissolved in 3 ml. of diethyl ether and treated with 0·1 ml. of the alkaline hydroxylamine. After addition of ether and alkaline hydroxylamine all the samples and standards of a batch were evaporated to dryness together in a water bath at 65°. They were left in the water bath for 5–7 min. and then cooled. Butanolic ferric perchlorate was added from a burette and the colours were read after 30–40 min.

Determination of nitrogen. Amino nitrogen was determined by the method of Cocking & Yemm (1954). Total nitrogen was determined by direct nesslerization after Kjeldahl digestion.

Determination of choline. Samples were hydrolysed under the conditions of Wheeldon & Collins (1958). The acid hydrolysate was extracted with light petroleum (b.p. 40–60°) to remove fatty acids and evaporated to dryness at 90° under a stream of N_2 to remove HCl. The residue was resuspended in 1–2 ml. of water and centrifuged. A portion of the clear supernatant, containing 0·02–0·6 μmole of choline, was used in the estimation by the following modification of the method of Appleton, La Du, Levy, Steele & Brodie (1953).

The aqueous choline solution (0·5 ml.) in a thick-walled glass centrifuge tube (5 cm. long, 5 ml. volume) was treated at 0° with one drop of a 5% Teepol solution and 0·5 ml. of the KI_3 reagent. After being stirred with a glass fibre the solution was left in ice for 30 min.

The tubes were capped and centrifuged at 0° (25 000 *g*, 45 min.). The supernatant was decanted and the tubes were inserted upside down into close-fitting celluloid

centrifuge tubes, the upper parts of which had been cut off. They were centrifuged in the upside-down position at 0° (200 *g*, 20 min.). The open ends of the tubes were wiped with filter paper to remove the last traces of KI_3 and the precipitates of choline iodide (choline I_9) were dissolved in redistilled ethylene dichloride. Extinctions were measured against ethylene dichloride at 365 mμ after about ½ hr. at room temperature in glass cells with a 2 mm. light-path. Choline chloride was used as standard.

The standard curve was linear in the region tested (0·02–0·8 μmole of choline chloride). It passed through the origin, indicating a complete removal of KI_3 from the precipitate. The colour densities of the ethylene dichloride solutions of choline I_9 were somewhat variable from one estimation to another. A set of standards was therefore included in each batch of samples.

Aldehydes and cholesterol. Aldehydes were measured by the method of Gray & Macfarlane (1958) and cholesterol (total) was determined by the method of Zlatkis, Zak & Boyle (1953).

Treatment of tissue

Rats (300 g. wt., Wistar strain) were killed by decapitation. The brains were immediately removed and minced in a stainless-steel Fisher mincer (Jouan, Paris). Approximately 1 g. of the mince was taken for direct saponification of the tissue lipids with ethanolic KOH (see below: 'Preparation of methyl esters').

The mince (12 g.) was homogenized in 4 vol. of sucrose (0·25 M) in a stainless-steel Potter–Elvehjem homogenizer (vol. 40 ml.) with the pestle revolving at 2000 rev./min. The homogenizer was moved up and down four times.

A volume of the homogenate containing 5 g. (wet wt.) of brain was removed for extraction of lipids. A further 2 vol. of sucrose (0·25 M) was added to the remaining homogenate. This was fractionated according to the scheme in Fig. 1, suggested by Dr D. E. Bellamy and similar to his published procedure (Bellamy, 1959).

Extraction of lipids

The mitochondrial and microsomal pellets and whole homogenate were separately homogenized in a MSE homogenizer with 10 vol. of $CHCl_3$–methanol (2:1, v/v). After 2 hr. at room temperature the material was filtered through a fat-free filter paper.

The filter paper containing the residue was re-homogenized with a further 10 vol. of $CHCl_3$–methanol (2:1, v/v), left for 2 hr. and the solid material filtered off and discarded. To the combined extracts was added one-fifth of their volume of NaCl (0·29 M). After shaking, the emulsion was left overnight at room temperature to separate the two phases. The upper (aqueous) phase was removed and the walls of the vessel and surface of the lower phase were washed twice with 'upper-phase liquid' [aq. 0·58% NaCl–methanol–$CHCl_3$ (47:48:3, by vol.); cf. Folch, Lees & Sloane-Stanley, 1957].

Insoluble 'lipoprotein' material at the interface (Folch, Ascoli, Lees, Meath & Le Baron, 1951) was removed and treated separately (see 'Treatment of lipoprotein').

Methanol was added to the washed lower phase until one phase resulted and the solution was evaporated to dryness at 45° in a stream of N_2. The residue was extracted with 2% methanol in $CHCl_3$ and the extract containing 'total lipids' was filtered and stored at −15°.

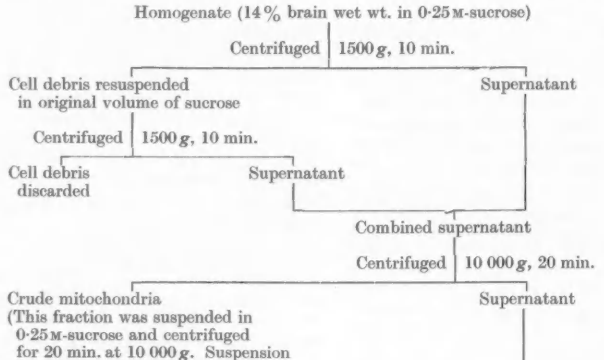
Homogenate (14% brain wet wt. in 0·25 M-sucrose)
Centrifuged
1500 g, 10 min.
Cell debris resuspended in original volume of sucrose
Supernatant
Centrifuged
1500 g, 10 min.
Cell debris discarded
Supernatant
Combined supernatant
Centrifuged
10 000 g, 20 min.
Crude mitochondria (This fraction was suspended in 0·25 M-sucrose and centrifuged for 20 min. at 10 000 g. Suspension in 0·25 M-sucrose and centrifuging were repeated three times)
Supernatant
Mitochondria
Combined washings
Combined supernatant
Centrifuged
25 000 g, 1 hr.
Microsome fraction
Supernatant discarded

Fig. 1. Procedure for isolation of rat-brain mitochondrial and microsomal fractions. All operations were carried out at 0–2° immediately after the death of the animal.

Table 1. *Eluting system employed for silicic acid-column chromatography of rat-brain lipids* (100–200 *mg.*)

Fraction no.	Composition of eluent	Vol. of eluent (ml.)	Expected composition of fraction
1	1% of diethyl ether in light petroleum	350	Steroid esters
2	10% of diethyl ether in light petroleum	500	Triglycerides, free cholesterol
3	Diethyl ether	300	Monoglycerides, diglycerides
4	Chloroform–methanol (7:1, v/v)	100	Polyglycerophosphatide etc. (unknown)
5	Chloroform–methanol (4:1, v/v)	200	Ethanolamine phosphatides, serine phosphatides
6	Chloroform–methanol (3:2, v/v)	100	Phosphoinositide
7	Chloroform–methanol (3:2, v/v)	400	Lecithin
8	Chloroform–methanol (1:4, v/v)	200	Sphingolipids
9	Methanol	400	Unknown lipids

Treatment of lipoprotein

The residues in the flask and on the filter from 'total lipid' extraction were combined and extracted with $CHCl_3$–methanol (1:1, v/v) (Folch *et al.* 1951). To this extract was added 'lipoprotein' from the interface of total lipid washing (above); the suspension was evaporated to dryness under N_2 and the residue was saponified.

Analysis of total lipid extracts

Extracts of mitochondria, microsomes and homogenate were analysed for lipid dry weight, total phosphorus and total acyl glyceryl ester of the original lipid. Extracts of mitochondria and microsomes were also analysed for total nitrogen, amino nitrogen, choline, aldehyde and total cholesterol. A portion of mitochondrial and microsomal extracts was taken for direct saponification and fatty acid analysis.

Separation of total lipids into lipid classes

For more detailed analysis total lipids were chromatographed on a silicic acid (100 mesh, Mallinckrodt Chemical Works, N.Y., U.S.A.) column prepared as described by Hirsch & Ahrens (1958). A system of stepwise elution based on systems of Hirsch & Ahrens (1958) and Hanahan, Dittmer & Warashina (1957) was used.

Lipids (100–200 mg.) were introduced on the column in light petroleum (b.p. 60–80°). The eluting solvents and expected composition of eluates are shown in Table 1. Eluted fractions were evaporated to dryness under a stream of N_2 and redissolved in $CHCl_3$.

Analysis of fractions from silicic acid columns

Paper chromatography. Phospholipid fractions were chromatographed at 0° on silicic acid-impregnated paper with di*iso*butyl ketone–acetic acid–water (40:20:3, by vol.) (Marinetti, Erbland & Kochen, 1957). Another solvent described by Marinetti *et al.* (1957), di*iso*butyl ketone–*n*-butyl ether–acetic acid–water (20:20:20:3, by vol.) gave poor separation.

Non-phosphatides were chromatographed at 23° on silicic acid-impregnated paper with *n*-heptane–di*iso*butyl ketone (96:6, v/v) (Marinetti, Erbland & Stotz, 1958).

The following reagents were used on a single chromatogram in the order shown: Rhodamine-6 G (fluorescent lipid spots, Marinetti *et al.* 1957); dipicrylamine for choline (Beiss & Armbuster, 1958); acid ammonium molybdate for phosphorus (Hanes & Isherwood, 1949). Colour was developed by heating and u.v.-irradiation.

A duplicate chromatogram was sprayed with ninhydrin to detect lipids containing amino groups.

R_F values of identified lipids were in the order given by Marinetti *et al.* (1958).

Chemical estimations. All fractions were analysed for acyl glyceryl ester, total phosphorus and amino nitrogen. Phospholipid fractions and mitochondrial Fraction 2 (Table 1) were analysed for aldehyde. Choline, cholesterol and total nitrogen were determined in those fractions where their presence was indicated by paper chromatography.

Preparation of methyl esters for gas–liquid chromatography

The fatty acids of all lipid fractions were analysed by gas–liquid chromatography of their methyl esters, which were prepared by one of two methods.

Diazomethane method. Lipids were saponified for 3 days at 30° with 2N-KOH in aq. 50% (v/v) ethanol containing 0·1% (w/v) of quinol as antioxidant. The unsaponifiable lipids were removed by shaking with light petroleum (b.p. 40–60°) and fatty acids were extracted into light petroleum after acidification of the mixture. Fatty acid extracts were washed with water, dried over Na_2SO_4 and esterified with diazomethane. This procedure was applied to the lipids of whole-brain mince, total lipid extracts of microsomes and mitochondria and proteolipids from whole-homogenate mitochondria and microsomes. Silicic acid fractions of homogenate were treated similarly, but extraction of unsaponifiable material was omitted, since the methyl esters were purified by sublimation (see below).

Interesterification with methanolic hydrochloric acid. This procedure, described by Stoffel, Chu & Ahrens (1959), was applied to silicic acid fractions of mitochondria and microsomes. All methyl esters were further purified by sublimation (Stoffel *et al.* 1959).

Gas–liquid chromatography

Treatment of sample. The instruments used were a chromatograph fitted with an ionization detector (Lovelock, 1958), built by Dr G. S. Getz and Mr A. Renshaw in

this laboratory, and an Argon Chromatograph (Pye Co. Ltd., Cambridge, England). Columns were 4 ft. long and 4 mm. in diameter. The solid supports used were Celite 545 (mesh 100–120) or Embacel (May and Baker Ltd., Dagenham, Essex). Apiezon L high-vacuum grease (20%) was used as non-polar stationary phase and polyethylene glycol adipate or polyethylene glycol adipate cross-linked with pentaerythritol (Cambridge Industries Ltd., Cambridge, Mass., U.S.A.; stationary phase LAC-2-R-446; 10 or 20%) were used as polar stationary phases. Column temperatures were between 175° and 195° and did not vary significantly during analysis.

Samples (0·05–0·1 μl.) of dry methyl esters were introduced by means of a Pye micropipette. Smaller samples were introduced with the ground end of a glass rod. For introducing the sample the flow of argon through the column was stopped and the pressure at the top of the column was allowed to drop to atmospheric. The pipette was introduced through a stopcock at the top of the column. Immediately after discharge of the sample on the stationary phase the pipette was withdrawn, the stopcock closed and gas flow resumed.

Calculation of proportions of methyl esters. Areas of peaks, on a recording of detector potential against time, were approximately proportional to mass of component eluted in the peak. The areas were measured by drawing the base line, and the tangents to the peak at the point of inflexion (Fig. 2, AB, BC), and measuring the area of triangle ABC (Keulemans, 1957).

Distances from the intersection of tangents B to peak *X* (due to resumption of gas flow through detector) were measured.

Retention volumes were expressed relative to stearate.

Identification of peaks. Fatty esters were identified by the following methods. (1) Comparison of their relative retention volumes with those of authentic samples of easily available esters and with data of James & Martin (1956) and Insull & Ahrens (1959) for stationary phases of similar polarity. (2) Hydrogenation and bromination (James & Martin, 1956) of unsaturated esters and gas chromatography of the products.

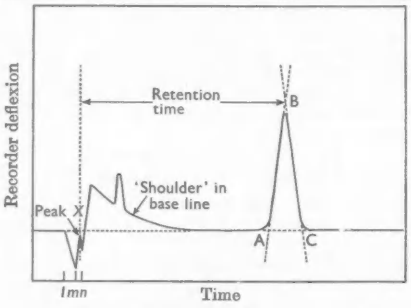

Fig. 2. Quantitative interpretation of gas chromatograms. Area ABC is proportional to mass of methyl ester eluted in the peak. During introduction of sample flow of argon is discontinued at *l* and resumed at *m*. Peak *X*, from which retention times were measured, corresponds to the beginning of negative deflexion *n* due to passage through the detector of air introduced together with the sample.

EVALUATION OF METHODS

Precautions against oxidation of lipids. Exposure of dry lipid material to atmospheric O_2 was kept to a minimum. Lipid was never left for long periods without organic solvent. It was stored at −15° dissolved in solvent. Solvent was removed by evaporation at 45–50° under a stream of dry O_2-free N_2. The experiment described below indicates the adequacy of these precautions.

An extract of sheep-heart lipids containing large proportions of polyunsaturated fatty acids was evaporated to dryness under O_2. It was saponified with 2N-KOH in aq. 50% ethanol without antioxidant in an atmosphere of O_2. The fatty acids were isolated and methylated with diazomethane. The solution was evaporated to dryness and the methyl esters were heated for 30 min. at 70° in a stream of O_2. Another portion of the same extract was saponified and methylated with diazomethane as described above ('Preparation of methyl esters'). Chromatograms of fatty acid esters obtained by both methods showed no appreciable differences.

The experiment indicates that fatty acids detected by gas chromatography under the conditions described in the Experimental section are not significantly oxidized during isolation and methylation.

Lipid-extraction procedure. Comparison of recoveries of methyl esters after extraction of brain mince with $CHCl_3$–methanol and after direct saponification indicated complete extraction of (fatty) acyl glyceryl esters. The methods of analysis used were unsuitable for the detection of cerebrosides or small amounts of sphingomyelin.

Silicic acid fractionation. Recoveries of various functional groups after fractionation are shown in Table 2. The figures may be in error by ±10% since minimum

Table 2. *Recovery of cholesterol, total phosphorus, total nitrogen and functional groups from silicic acid fractionation*

$$\text{Percentage recovery} = \frac{100 \times \Sigma \text{ moles of component in fractions analysed}}{\text{moles of component in total-lipid extract of same tissue fraction}}.$$

Tissue fraction	Percentage recovery						
	Total phosphorus	Acyl ester	Total nitrogen	Amino nitrogen	Choline	Aldehyde	Cholesterol
Homogenate	103	108	*	*	*	*	*
Mitochondria	96	123	86	111	81	93	17
Microsomes	93	93	93	105	97	63†	14

* No analyses were made for these on total-homogenate extract.

† The apparent low recovery is probably an artifact of aldehyde estimation (see Evaluation of Methods section).

Table 3. *Elution pattern of brain lipids*

Mitochondrial Fraction 3 was lost. Fraction 9 (100% methanol) was collected only from homogenate lipids. Serine–ethanolamine–inositol phosphatide fraction of homogenate also contained 'polyglycerophosphatide'. For composition of eluents used for separating the Fractions and their expected lipid contents see Table 1.

Lipid class	Silicic acid fractions in which lipid classes were isolated: Homogenate	Mitochondria	Microsomes
Non-phospholipids (mainly 'neutral' lipids, fatty acids)	1 2 3	1 2 .	1 2 3
Polyglycerophosphatide	Not isolated	4	4
Serine phosphatides	4	5	5
Ethanolamine phosphatides	5	6	.
Inositol phosphatides	6	.	.
Lecithin	. 7	. 7	6 7
Polar lipids (unidentified)	8 9	8 .	8 .

Table 4. *Percentage recovery of ester groups after methylation and sublimation*

$$\text{Percentage recovery} = \frac{100 \times [\text{methyl ester in sublimate (moles)}]}{[\text{acyl glyceryl ester in original lipids (moles)}]}$$

For expected composition of silicic acid fractions see Table 1. For composition of fractions as isolated see Table 2.

Tissue fraction	Silicic acid fraction no.	Recovery (%)
Homogenate	Total extract	Not esterified
	1	590
	2	219
	3	114
	4	102
	5	108
	6	102
	7	80
	8	124
	9	580
Mitochondria	Total extract	103
	1	89
	2	78
	3	Not esterified
	4	55
	5	92
	6	97
	7	147
	8	120
Microsomes	Total extract	87
	1	37
	2	121
	3	74
	4	60
	5	71
	6	97
	7	96
	8	170

amounts of material were used for analysis. The recoveries appear sufficient for the fractionated lipids to be considered representative of original lipids.

The recovery of cholesterol was low (15%). Cholesterol esters, however, constitute only 2–3% of non-phospholipids by weight (Williams *et al.* 1945), so that the poor recovery is not likely to affect fatty acid distribution significantly. Silicic acid fractionation did not separate all lipid classes completely. Sphingomyelin, if present, was eluted with lecithin. In calculating the results analyses of eluate fractions were combined in groups to give analytical data for the major lipid class present in those fractions (see Table 3). The minor components were treated as contaminants. The differences in elution pattern between tissue fractions (Table 3) are probably caused by the small differences in fatty acid composition found.

Preparation of methyl esters for gas chromatography. When esterification was carried out with diazomethane, peak E (Table 5) appeared in trace quantities on chromatograms on polar stationary phase. The peak was not observed in esters prepared by prolonged methanolysis (Stoffel *et al.* 1959). It is probably the product of a side reaction of diazomethane.

Table 4 shows the recoveries of ester groups after methylation and sublimation. The too-high recoveries in homogenate neutral lipids (Fractions 1–3) and in polar lipids (Fractions 8 and 9) will be referred to in the discussion of results for these lipids. Polyglycerophosphatide fractions and microsomal kephalin fraction gave very low recoveries of methyl esters (60 and 70% respectively). Other fractions gave yields approaching 100%.

The methods of preparation and purification of methyl esters used do not separate them from aldehydes resulting from plasmalogen hydrolysis. As a result aldehyde peaks appear on gas chromatograms.

Identification of peaks in gas–liquid chromatography. Identification of peaks and their retention volumes relative to stearate are shown in Table 5. For minor unsaturated or branched esters assignment of structure is based upon the comparison of their retention volumes with other peaks.

Table 5. *Identification and retention volumes of compounds appearing on gas chromatograms*

*, Indicates tentative identification; un., unsaturated; br., branched. Chain $n-x$ contains n C atoms and x double bonds [notation after Ahrens *et al.* (1959)].

$n-x$	Description or trivial name	Retention volume relative to methyl stearate: Apiezon L	Retention volume relative to methyl stearate: Polyethylene glycol adipate
	Esters		
8–0*	.	0·015	.
10–0*	.	0·035	.
12–0	Lauric	0·076	0·103
12– or 13–un.*	.	.	0·135
13–0	.	0·128	0·150
13–un.*	.	0·081	0·196
14–0	Myristic	0·186	0·230
14–un.*	.	0·170	0·235
14–1*	.	0·160	0·263
15–0	.	0·307	0·339
15–1*	.	0·251	0·394
16–0	Palmitic	0·435	0·501
16–1	Palmitoleic	0·380	0·560
17–br.*	.	0·499	0·695
18–0	Stearic	1·00	1·00
18–1	Oleic	0·862	1·11
18–2	Linoleic	0·801	1·34
19–0*	.	1·64	.
20–0*	Arachidic	2·32	2·02
20–1	.	1·95	2·17
20–4	Arachidonic	1·49	3·25
22–5*	.	3·25	6·61
22–6*	.	2·99	8·73
C	Unidentified highly polar components	0·186	1·62
D		0·435	2·83
E	Unidentified	.	6·05
F	Unidentified	1·10	.
G	Unidentified	1·34	.
	Aldehydes		
H	Unidentified	0·289	0·409
A	Unidentified	0·545, 0·562, 0·602	0·790
B	Unidentified	0·657, 0·688, 0·717, 0·747	0·867

Whereas the use of two stationary phases of widely different polarities is an aid to identification, the structures given for these peaks (marked with an asterisk in Table 5) are only tentative. This also applies to peaks 8–0, 10–0 and 19–0, which were detected only on one stationary phase, and to peak 20–0, which was a trace component of one of the fractions.

Unidentified components in gas–liquid chromatography. Peaks A, B and H are due, at least partly, to aldehydes. The nature of the aldehydes has not yet been elucidated. It is possible that some of these peaks also contain a methyl ester.

On non-polar stationary phase the relative retention volumes of peaks A and B varied from sample to sample more than those of other peaks. A possible explanation for this variability is a partial reaction of diazomethane with the aldehydes during methylation of esters in the sample. These may run with retention volumes different from original aldehydes.

Peaks C and D, due to highly polar compounds, are discussed under Results ('Neutral lipids').

Peak E is a trace component, probably produced by interaction of diazomethane with a double bond (see above, 'Preparation of methyl esters').

Peaks F and G are unidentified trace components of some fractions.

Quantitative estimation of esters by gas–liquid chromatography. Fatty acid-distribution figures are calculated as:

$$\text{fatty acid (\%)} = \frac{100 \times (\text{peak area of component})}{\Sigma\ (\text{peak area of all measured components})}.$$

Thus components detected but not measurable because of small or overlapping peaks were neglected.

In most samples the sum of measurable peaks accounted for over 95% of esters present. In those fractions where only very small samples were available for gas chromatography (polar lipids, some neutral-lipid fractions), measured peaks may represent a much smaller fraction of total esters. This source of error does not affect the ratios between compounds with measurable peaks on any single chromatogram.

The accuracy and reproducibility of gas chromatography were not critically examined, but some general observations are given. Short-chain acids (C_8–C_{14}), which gave very narrow peaks lying in some chromatograms on a shoulder (see Fig. 2), and long-chain acids (C_{22}) with long retention times, which gave low broad peaks, are estimated less accurately than acids in the C_{16}–C_{20} range. Small amounts of C_{23} and C_{24} acids would probably not have been detected at the temperature and flow rates used.

For well-resolved esters containing 20–30% of total ester groups the error in the percentage figure due to the above causes may be up to 12% of that figure and for esters containing 2% of total ester groups up to 100% of the figure (i.e. from trace quantity to 4%). More exact figures are given, since peaks of similar magnitude, especially within the C_{16}–C_{20} chain-length range, can be compared with a much higher degree of accuracy.

Small amounts of acids with retention volumes similar to those of major components were difficult to estimate accurately (palmitoleate, linoleate, 22–5). Inadequate resolution may also interfere with estimation of major peaks. Thus oleate was overestimated by about 10% of its content on the polyethylene glycol adipate column, on which it was inadequately resolved from preceding stearate.

Chemical estimations. (*a*) Choline estimation. In the silicic acid fractions containing only lecithin and/or kephalin the ratios choline/phosphorus or (choline + amino nitrogen)/phosphorus exceeded 1. On this basis it was assumed that choline was over-estimated by 10–15%.

The overestimation is attributed to coprecipitation of water-soluble organic material with the choline I_2. The contaminating material can trap KI_3 during centrifuging and give additional colour in ethylene dichloride solution.

(*b*) Aldehyde estimation in microsomal Fraction 5. About 50% of the aldehyde–rosaniline complex was not extracted into octan-2-ol from the aqueous layer. The effect was not observed with total microsomal lipids, where all the rosaniline complex was extracted into the octan-2-ol phase.

It is assumed that all microsomal aldehyde was in fact recovered in the kephalin fraction, as was the case in mitochondrial lipids.

RESULTS

Whole-brain analysis

Tissue fractionation. The yield of mitochondria was 20·2 mg. dry wt./100 mg. dry wt. of homogenate and the yield of microsomes 20·5 mg. The procedure used aimed at purity of subcellular organelle fractions rather than maximum yields. In particular a part of the mitochondrial fraction was discarded with the cell debris. Of the mitochondrial pellet 15% (by vol.) consisted of nuclei and cell debris. Of the microsomal pellet 10% (by vol.) consisted of mitochondria and unidentified small vesicles. (These data were kindly supplied by Dr D. E. Bellamy.)

Lipid extraction. Table 6 shows the content of some constituents and functional groups in the total lipids. On the basis of these data approximate contents of different lipids by weight are given in Table 7 together with the total lipid dry weight. Mitochondria have the highest content of phospholipids and neutral lipids.

The recoveries of total lipids as well as of individual lipid classes are substantially lower than those obtained by Williams *et al.* (1945) from brains of 70-day-old rats (Table 7). Differences in animals and technique make the significance of these lower yields difficult to assess. Low yield of phospholipids may be partly due to lack of recovery of sphingomyelin.

Table 6. *Content of total phosphorus, cholesterol and neutral lipids in total-brain lipids*

	Concentration (μmoles/100 mg. dry wt. of tissue)		
	Total phosphorus	Cholesterol	Neutral lipids (esters of fatty acids after methylation)
Homogenate	30·6	Not analysed	2·79
Mitochondria	34·6	14·8	12·0
Microsomes	32·2	17·1	3·51

Table 7. *Recoveries of lipids calculated on basis of analyses in Table* 6

For comparison, recoveries obtained by Williams *et al.* (1945) for brain lipids of 70-day-old rats are given. Total lipid dry weights are maximal since the lipid extracts on which they were determined may have contained sucrose from the homogenizing medium, which was not removed by washing the lipid extracts.

Phospholipid dry weight was calculated from the phosphorus content by using an arbitrary molecular weight of 750 (mean of distearyl phosphatidylethanolamine and distearyl lecithin).

Neutral fat was calculated as grycerol tristearate on the basis of sublimed methyl esters (to include free fatty acid content).

	Concentration (mg./100 mg. dry wt. of tissue)							
	Total lipids (dry wt.)		Phospholipids		Cholesterol		Neutral fat	
	Present study	Williams *et al.*	Present study	Williams *et al.*	Present study	Williams *et al.*	Present study	Williams *et al.*
Homogenate	39·5	44·63	22·9	27·19	Not determined	7·05	0·8	1·95
Mitochondria	51·7	.	25·8	.	6·0	.	3·6	.
Microsomes	34·2	.	22·2	.	6·6	.	1·1	.

The relatively high proportion of neutral lipids in mitochondria (3·6% of the dry weight) is a striking feature of the total lipid distribution. The major part of this lipid consists of the polar compounds 'C' and 'D' (see above: 'Identification of peaks in gas–liquid chromatography').

Composition of rat-brain phospholipids

General assumptions and interpretation of data. Amino-nitrogen content of a fraction is considered equivalent to the content in that fraction of serine and ethanolamine phosphatides (kephalins). Where the identity of the other component of a fraction is known, but its content has not been directly measured, its proportion is expressed as its phosphorus content; thus 'moles of inositide P in kephalin fraction = moles of total P in fraction − moles of amino N'.

Lysophosphatides are calculated from the following equation:

moles/100 moles of total lipid P contained in lysophosphatides
= 2[moles of total P − (moles of acyl glyceryl ester + moles of aldehyde)].

This is a very rough approximation, since the figure obtained is a small difference of two large numbers. The lysophosphatide content may be in error by 50% for some fractions.

Plasmalogen is calculated on the basis of one ether linkage per molecule.

Content of individual lipid classes. Table 8 shows the composition of total phospholipids based upon analyses of total extracts and of tissue fractions. The proportion of lecithin found is similar to the figure given by Williams *et al.* (1945) for a 70-day-old brain. Brain appears to contain less lecithin in phospholipids than do other rat organs (Williams *et al.* 1945), ox heart (Gray & Macfarlane, 1958) or pig heart (Marinetti *et al.* 1958). The differences are of the order of 10–15% of total phospholipid phosphorus.

Polyglycerophosphatide and inositol phosphatide were not measured directly. The figures given are approximations. The proportion of polyglycerophosphatide in brain mitochondria is considerably smaller than in other organs [cf. ox heart (Gray & Macfarlane, 1958), 9% of phospholipid phosphorus; rat-liver mitochondria (Getz & Bartley, 1959), 10%; pig-heart mitochondria (Marinetti *et al.* 1958), 8%]. The polyglycerophosphatide also differs in its fatty acid structure.

The content of mitochondrial inositol phosphatide was similar to that of pig-heart mitochondria [Marinetti *et al.* (1958), 14%]. In microsomes it was much higher than in pig-heart microsomes (2%). Gray & Macfarlane (1958) give a somewhat lower figure for ox-heart mitochondria (6%) and higher for ox-heart microsomes (10%) than Marinetti *et al.* (1958).

Lipid distribution in mitochondria and homogenate is very similar. Microsomes have a higher proportion of lecithin with respect to kephalin. The composition of brain phospholipids obtained in the present study differs considerably from that given by Petersen & Schou (1955).

An unusual feature is the large excess of total nitrogen over the sum of nitrogeneous bases (choline + serine + ethanolamine) in all fractions. The nature of compounds containing this nitrogen is unknown. Since proteolipids were not rigorously

Table 8. *Composition of brain phospholipids calculated on a molar basis from analyses of total extracts and silicic acid-chromatography fractions*

'Serine and ethanolamine phosphatides' ≡ total amino-N content. 'Lecithin' ≡ phosphorus − amino N in lecithin fraction. Polyglycerophosphatide ≡ phosphorus − amino N in polyglycerophosphatide fraction. Inositol phosphatide ≡ phosphorus − amino N in 'kephalin' fraction. (Polyglycerophosphatide + inositol phosphatide) ≡ (phosphorus − amino N in 'kephalin' fraction) [in homogenate only]. Plasmalogen = aldehyde content (after hydrolysis). Lysophosphatides ≡ 2[(total phosphorus) − (acyl glyceryl ester + aldehyde)].

	Composition (moles/100 moles of phospholipid P)		
	Homogenate	Mitochondria	Microsomes
Acyl glyceryl ester (in lipids before methylation)	175	181	170
Serine and ethanolamine phosphatides	48·0	50·0	41·4
Lecithin	31·4	32·1	38·9
Polyglycerophosphatide	.	1·6	2·2
Inositol phosphatide	.	13·4	12·2
Inositol + polyglycerophosphatide	17·8	.	.
Polar lipids fraction	4·0	0·5	1·4
Plasmalogen	14·4	16·8	16·1
'Lysophosphatides'	Not determined	15·2	13·2
Total N − (amino N + choline)	75	50	68

Table 9. *Fatty acid distribution in total-brain lipids*

Chain $n-x$ contains n C atoms and x double bonds. br., Branched.

$n-x$	Trivial name	Methyl ester in measurable peaks (%)		
		Saponified whole brain	Saponified extracts	
			Mitochondria	Microsomes
	Esters			
13–0	.	–	1·8	2·5
14–0	Myristic	–	0·5	1·5
14–1	.	–	0·6	1·2
16–0	Palmitic	23·9	28·4	30·7
16–1	Palmitoleic	1·3	1·0	0·5
17–br.	.	–	–	1·7
18–0	Stearic	22·8	22·7	24·5
18–1	Oleic	30·0	31·9	24·0
18–2	Linoleic	1·4	–	–
20–1	.	3·7	1·6	1·4
20–4	Arachidonic	9·5	7·1	6·8
22–6	.	7·6	4·3	6·8
	Trace components (esters)			
10–0	.	+	+	–
12–0	Lauric	+	+	+
	Summation of esters			
$n-0$	.	46·7	50·5	59·3
$n-1$	.	34·5	34·5	25·9
$n-2$	.	1·4	–	–
$n-x>2$	.	17·0	11·4	13·5
	Aldehydes			
H, A, B	See Table 5	2·2; 3·3; 3·1	1·0; 2·2; 1·9	1·6; 1·1; 3·1

Table 10. *Distribution of fatty acids in proteolipids*

Chain $n-x$ contains n C atoms and x double bonds. Proteolipids in column II were extracted by the procedure given in the Experimental section from a different batch of rat brains without homogenization with sucrose before extraction.

$n-x$	Description or trivial name	Methyl ester in measurable peaks (%)			
		Homogenate extract (I)	Whole-brain extract (II)	Mitochondria extract	Microsomes extract
	Esters				
14–0	Myristic	+	+	0·3	+
16–0	Palmitic	25·9	26·6	26·3	29·8
16–1	Palmitoleic	1·1	1·3	0·6	0·5
18–0	Stearic	23·8	25·3	20·9	22·4
18–1	Oleic	31·7	32·0	29·5	24·0
18–2	Linoleic	+	+	3·9	3·6
20–1	.	3·8	–	3·1	1·8
20–4	Arachidonic	7·6	6·9	7·4	7·7
22–6	.	6·1	7·8	8·0	10·0
	Trace components (esters)				
12–0	Lauric	+	+	+	+
15–0	.	–	+	+	–
G	.	–	–	+	–
	Summation of esters				
$n-0$	.	49·7	51·9	47·5	52·2
$n-1$	.	36·6	33·3	33·2	26·3
$n-2$	.	+	+	3·9	3·6
$n-x>2$	.	13·7	14·7	15·4	17·7
	Aldehydes				
H	.	2·0	2·9	1·7	1·9
A	.	5·0	0·9	2·5	1·6
B	.	5·0	4·5	–	–

removed from the extracts the nitrogen may derive from contaminating protein.

Fatty acid composition of total lipids of whole mince and subcellular fraction extracts is shown in Table 9. The general pattern of fatty acid distribution is similar in all three extracts. Mitochondria and microsomes contain slightly higher proportions of saturated fatty acids and less 'essential' acids (linoleic acid and polyenoic acids) than does the whole mince.

Brain lipids are remarkable for their high content of saturated acids and very low content of 'essential' fatty acids relative to other organs. Essential fatty acids were present in the diet (Dr G. S. Getz, unpublished work).

Comparison of particular brain lipids with lipids found in other organs will be made in the discussion of lipid fractions.

Fatty acid composition of 'proteolipids' is shown in Table 10. The distribution of fatty acids in all the proteolipids closely resembles distribution in the corresponding total lipids. In view of the isolation by Folch *et al.* (1951) of proteolipids in crystalline form with specific proportions of lipid and protein a greater specificity of fatty acid distribution might be expected. It is possible, however, that the proteolipid fraction isolated in the present experiment contains a large proportion of 'total' lipids occluded on protein during extraction.

In a separate experiment the influence of age on total brain fatty acids was studied by saponification of whole mince or whole-lipid extracts from brains of animals at different ages (Table 11).

The brain fatty acids of 5-day-old rats contained as much as 48 % of palmitic acid, and a total of 60 % of saturated acids. The proportion of palmitic acid decreases with age to 21 % in the adult animal (cf. also 'whole brain' in Table 9) whereas the proportions of stearic acid and oleic acid increase.

Since lecithin is the component lipid richest in palmitic acid (cf. Table 14), it would be interesting to find out whether the high proportion of palmitic acid in young brain correlates mainly with an increased lecithin content (as found by Williams *et al.* 1945) or whether it reflects a general change in fatty acid composition.

Distribution of fatty acids within individual lipid classes. (*a*) Kephalin fraction (kephalin comprises serine and ethanolamine phosphatides). Paper chromatography indicated the presence in this fraction of amino-nitrogen-containing lipids with R_p values corresponding to phosphatidylserine and phosphatidylethanolamine, lysophosphatides, and of non-nitrogenous phosphatides with R_p values similar to phosphoinositide (Marinetti *et al.* 1958).

Table 11. *Influence of age on brain lipids*

Chain $n-x$ contains n C atoms and x double bonds.

		Methyl ester in measurable peaks (%)		
$n-x$	Trivial name	Saponified whole brain (adult)	Lipid extract of whole brain (22 days old)	Saponified whole brain (5 days old)
	Esters			
14–0	Myristic	1·0	0·5	–
14–1	.	–	–	2·9
16–0	Palmitic	23·4	32·6	48·2
16–1	Palmitoleic	1·2	2·4	6·3
18–0	Stearic	24·9	27·5	16·6
18–1	Oleic	30·9	21·8	15·1
18–2	Linoleic	–	–	1·3
20–1	.	4·6	–	–
20–4	Arachidonic	8·4	11·2	9·7
22–6	.	5·6	3·8	–
	Trace components (esters)			
18–0	.	–	+	–
22–5	.	–	+	–
	Summation of esters			
$n-0$	.	49·4	60·8	64·7
$n-1$	.	37·9	24·2	21·4
$n-2$	.	–	–	1·3
$n-x>2$	.	14·0	15·0	13·5
	Aldehydes			
H	See Table 5	2·7	3·8	2·8
A	See Table 5	3·1	0·9	–
B	See Table 5	4·2	3·9	0·9

The recovery of amino nitrogen and the components present in the fractions are shown in Table 12.

The recovery of amino nitrogen (85–100 %) is considered sufficient to give a representative fatty acid distribution.

Kephalin fraction contains a large amount of phosphoinositide. Assuming equal distribution of lyso compounds between nitrogenous phospholipids and phosphoinositide, the latter accounts for approx. 30 % of fatty acids in this fraction in whole homogenate, 20 % in mitochondria and 25 % in

Table 12. *Composition of kephalin fractions on a molar basis*

Lecithin ≙ choline in fraction. (Inositol + polyglycerophosphatide) ≡ Total P − (amino N + choline). Plasmalogen ≡ aldehyde. Lysophosphatide ≡ 2[(total P) − (glyceryl acyl ester + aldehyde)].

	Homogenate	Mitochondria	Microsomes
Recovery (% of total amino N recovered in fraction)	95·2	98·8	83·3
	Composition (moles/100 moles of amino N)		
Total P	146	128	135
Glyceryl acyl ester	218	200	209
Lecithin	2·8	2·2	0
Inositol and polyglycerophosphatide	42·7	26·2	35·2
Plasmalogen	36·7	32·2	45·2 (28·1)*
Lysophosphatides	36	25	10
Total N − (amino N + choline)	126	81·2	79·2

* Figure in parentheses represents aldehyde actually measured. Other figure gives the plasmalogen content on the basis of estimation on total-microsome lipid extract.

Table 13. *Fatty acid distribution in kephalin fractions*

Chain $n-x$ contains n C atoms and x double bonds. un., Unsaturated; br., branched.

$n-x$	Description or trivial name	Methyl ester in measurable peaks (%) Homogenate	Mitochondria	Microsomes
	Esters			
13–0	.	+	0·3	0·9
14–1	.	+	+	0·4
16–0	Palmitic	7·1	8·7	9·3
16–1	Palmitoleic	1·7	1·0	–
18–0	Stearic	34·3	32·4	41·8
18–1	Oleic	35·2	30·3	25·8
18–2	Linoleic	+	1·0	+
20–1	.	3·8	3·2	1·6
20–4	Arachidonic	9·9	10·9	8·7
22–5	.	+	3·1	2·4
22–6	.	7·6	9·0	9·1
	Trace components (esters)			
8–0	.	+	–	–
10–0	.	+	+	–
12–0	Lauric	–	–	–
13–un.		–	–	+
14–0	Myristic	–	+	+
15–1	.	–	+	–
17–br.	.	+	+	–
19–0	.	+	–	–
C	Methylation (diazomethane) artifact	–	–	+
E, F, G	See Table 5	Unidentified	–	+
F	See Table 5	Unidentified	–	+
G	See Table 5	Unidentified	–	+
	Summation of esters			
$n-0$	.	41·8	41·4	52·0
$n-1$	.	40·7	34·5	27·4
$n-2$	.	–	1·0	–
$n-x>2$	.	17·6	23·0	11·1
	Aldehydes			
H	See Table 5	.	2·0	2·0	3·1
A	See Table 5	.	3·9	0·6	0·9
B	See Table 5	.	3·6	0·3	6·3

microsomes. As a result of this contamination, the fatty acid distribution in Table 13 is only a rough approximation to the distribution in brain kephalins.

All the aldehyde of total-lipid extracts was recovered in the kephalin fractions on silicic acid chromatography.

Anomalies in the aldehyde estimation in the microsomal fraction are discussed in the 'Evaluation of Methods' section. When allowance is made for relatively low recovery of amino nitrogen in the microsomal kephalin, plasmalogen content approaches that of the whole homogenate.

In contrast to the proportions of plasmalogen found for brain lipids, Gray & Macfarlane (1958) found in ox heart nearly 50% of kephalin and 60% of lecithin in the plasmalogen form. Even allowing for inaccuracies in the computation of lysophosphatides the figure obtained both in kephalin and lecithin fractions (see below) is surprisingly high. No comparable amount of fatty acids was detected (free fatty acids would cause yields of esters after methylation of over 100%). Incomplete recovery of methyl esters may, however, have obscured their presence.

Even if the lysophosphatide content was entirely due to breakdown of the lipids on the silicic acid column or on storage, only 18% of the fatty acids was lost in the homogenate, 13% in mitochondria and 5% in microsomes. Thus possible breakdown does not invalidate the results of gas–liquid chromatography, unless the breakdown is confined to one constituent lipid class or a narrow group of fatty acids.

Although the proportion of saturated fatty acids is only slightly lower than in total lipids there is a remarkably low proportion of palmitate relative to stearate in all three fractions. This has no parallel in the fatty acids of kephalin of rat-liver mitochondrial, microsomal (Macfarlane, Gray & Wheeldon, 1960) or whole-liver kephalin fatty acids (Dittmer & Hanahan, 1959).

The proportion of 'essential' polyene fatty acids is low in comparison with heart and liver, with only about 1% of linoleic acid, but the sum of the polyenes in mitochondrial and microsomal kephalin is about twice that in the whole lipids of the corresponding tissue fractions.

(*b*) Lecithin fraction. Paper chromatography of the lecithin fractions showed one strong, choline-positive spot and two other weak lipid spots, one with a higher R_F corresponding to phosphatidylserine or phosphatidylethanolamine and one with a lower R_F corresponding possibly to sphingomyelin. The weak spots were ninhydrin- and dipicrylamine-negative, but may have contained insufficient material for detection. Table 14 shows the composition of this fraction for the three tissue fractions.

The recovery of choline (85–100%) was sufficient to yield representative fatty acid samples. The composition is expressed as a percentage of moles of lecithin phosphorus (calculated as moles of total P − moles of amino N), since no significant amounts of other components were present on the basis of paper chromatography.

The contamination with kephalin is highest in homogenate lecithin, where the kephalin accounts for 20% of total fatty acids. Contamination in the other tissue fractions is much smaller. Only traces of aldehyde were detected. This is in sharp contrast with the results of Gray & Macfarlane (1958) for ox heart, where 60% of the lecithin contained plasmalogen.

It is possible that unsaturated ether linkages were hydrolysed on the silicic acid column in the presence of the polar solvents used to elute the kephalin fractions. Such hydrolysis would, however, be expected to occur earlier in the elution (cf. Gray & Macfarlane, 1958), giving rise to significant aldehyde recoveries from 'neutral fat' and polyglycerophosphatide fractions. In view of the absence of reports of lecithin plasmalogen in brain, this lipid class is not likely to be present in brain in large amounts.

The negative lysophosphatide content of the homogenate is a calculation artifact. The figures for the other two fractions are surprisingly high. They

Table 14. *Composition of lecithin fractions*

Lecithin P ≡ total P − amino N. Kephalin ≡ amino N. Plasmalogen ≡ aldehyde. Lysophosphatide ≡ 2[(total P) − (acyl glyceryl ester + aldehyde)].

	Homogenate	Mitochondria	Microsomes
Recovery (% of total choline recovered in fraction)	95·8	87·2	98·3
	Composition (moles/100 moles of lecithin P)		
Total P	128	107	116
Acyl glyceryl ester	282	184	210
Choline	111	116	114
Kephalin	27·7	6·7	15·8
Plasmalogen	0	0	1·0
Lysophosphatides	−26	30	21
Total N − total P	67	32	−5

Table 15. *Fatty acid distribution in lecithin fractions*

Chain $n-x$ contains n C atoms, x double bonds. un., Unsaturated; br., branched.

$n-x$	Description or trivial name	Methyl ester in measurable peaks (%) Homogenate	Mitochondria	Microsomes
	Esters			
12–0	Lauric	+	–	0·2
13–0	.	–	+	0·1
13–un.	.	–	+	0·2
14–0	Myristic	0·4	0·2	0·5
15–0	.	–	+	0·2
16–0	Palmitic	49·7	38·9	44·7
16–1	Palmitoleic	0·8	0·8	1·8
18–0	Stearic	12·8	17·1	15·9
18–1	Oleic	30·2	32·2	26·6
18–2	Linoleic	0·9	0·8	1·1
20–0	Arachidic	–	+	0·3
20–1	.	2·1	1·3	1·2
20–4	Arachidonic	3·3	4·7	4·1
22–6	.	+	3·6	2·6
	Trace components (esters)			
14–1	.	–	+	+
15–1	.	–	+	+
17–br.	.	–	+	+
22–5	.	–	+	+
	Summation of esters			
$n-0$	.	62·9	56·2	61·4
$n-1$	.	33·1	33·3	29·6
$n-2$	.	0·9	0·8	1·1
$n-x>2$	.	3·3	8·3	6·7
	Aldehydes			
H, A, B	See Table 5	.	–	+	0·3
		.	–	+	+
		.	+	0·2	0·2

are unlikely to represent lysolecithin content *in vivo*.

If the lysolecithin content given by the computation is correct and is entirely due to breakdown, the proportions of fatty acids lost amount to 15% in mitochondria and 10% in microsomes. The possible breakdown is not likely to affect significantly the fatty acid-distribution figures.

Fatty acid composition of brain lecithin is shown in Table 15. The distribution patterns in homogenate, mitochondria and microsomes are similar.

Lecithin shows the highest proportion of saturated acids of all lipid fractions. Some di-saturated lecithin is probably present, since saturated acids comprise over 50% of the total. Palmitic acid is present in high concentration (cf. kephalins, Table 13). In contrast with kephalin, in lecithin the proportion of 'essential' fatty acids in all tissue fractions is lower than in whole lipids.

The fatty acid distribution found in lecithin differs widely from that of whole-liver lecithin (Dittmer & Hanahan, 1959) or the lecithin of liver mitochondria and microsomes (Macfarlane *et al.* 1960). Both these groups found a much smaller proportion of saturated fatty acids (35% in liver mitochondria and microsomes) and a much higher proportion of linoleic acid and polyenoic acids (45% in liver mitochondria and microsomes).

The proportion of oleic acid (25–30%) is remarkably constant in the major phospholipids of each tissue fraction (cf. Tables 9, 11, 13 and 15).

Table 16. *Composition of polyglycerophosphatide fractions on a molar basis*

Polyglycerophosphatide P ≡ (total phosphorus) – (amino N). Polyglycerophosphatide ester (minimum) ≡ (acyl glyceryl ester) – 2(amino N). Kephalin ≡ amino N.

	Composition (moles/100 moles of polyglycerophosphatide P) Mitochondria	Microsomes
Acyl glyceryl ester	450	260
Kephalin	43	27
Polyglycerophosphatide ester / Polyglycerophosphatide P	3·6:1	2·0:1
Total N – amino N	213	No analysis

(*c*) 'Polyglycerophosphatide' fractions. Chromatography of these fractions on silicic acid-impregnated paper showed a fast-moving, ninhydrin-negative spot followed by a ninhydrin-positive spot with R_F corresponding to phosphatidylethanol-

Table 17. *Fatty acid distribution in polyglycerophosphatide fractions*

Chain $n-x$ contains n C atoms and x double bonds. un., Unsaturated; br., branched.

$n-x$	Description or trivial name	Methyl ester in measurable peaks (%)	
		Mitochondria	Microsomes
	Esters		
13–0	.	2·1	3·2
14–0	Myristic	0·8	+
14–un.	.	1·2	1·7
15–0	.	–	2·2
16–0	Palmitic	12·5	12·1
16–1	Palmitoleic	5·0	2·3
17–br.	.	1·0	+
18–0	Stearic	21·3	24·5
18–1	Oleic	18·6	21·6
18–2	Linoleic	10·3	2·3
19–0	.	+	0·8
20–0	Arachidic	1·8	1·3
20–1	.	1·3	1·6
20–4	Arachidonic	7·5	6·6
22–6	.	7·6	8·4
C, D	Unidentified, highly polar component	1·8, 6·9	1·8, 9·3
	Trace components (esters)		
8–0	.	+	–
12–0	Lauric	+	+
13–un.	.	–	+
14–un.	.	+	+
15–1	.	+	+
F	Unidentified	+	+
	Aldehydes		
H, A, B	See Table 5	+, +, +	1·4, 0·7, +

Table 18. *Composition of polar lipid fractions*

	Composition (moles/100 moles of total P)		
	Homogenate	Mitochondria	Microsomes
Acyl glyceryl ester	50	200	220
Amino N	16	Not analysed	57

amine. The fast-moving spot was not detected in other fractions. Since these fractions, however, had a much higher lipid content a substantial portion of the fast-moving spot may have been present in them and escaped detection. The R_F of the fast spot is consistent with it being polyglycerophosphatide.

The composition of the fraction (Table 16) is given as a percentage of polyglycerophosphatide phosphorus, calculated as total P–amino N. Owing to the high ester/P ratio of polyglycerophosphatide only 20% of acyl glyceryl esters in the fraction are derived from contaminating kephalins. Hence the fatty acid distribution in the fraction is unlikely to be considerably influenced by kephalin contamination.

Polyglycerophosphatide fractions from both mitochondria and microsomes gave very low recoveries of methyl esters after sublimation (55–60%, Table 4, Fraction 4). Distribution of fatty acids in polyglycerophosphatide fractions is shown in Table 17. The most interesting feature is the relatively high content of 'essential' fatty acids in mitochondria (25%) and microsomes (17%).

The polyglycerophosphatide fraction may be compared with the 'cardiolipin' fraction of Gray & Macfarlane (1958) from ox heart and of Getz & Bartley (1959) from rat liver with a linoleic acid content of 80%. Although the polyglycerophosphatide fraction is the most unsaturated brain lipid it does not correspond in its fatty acid composition to the cardiolipin fraction. Even if all the fatty acid 'missing' as a result of low yield from sublimation were linoleic acid (which is unlikely), the fraction would contain a smaller proportion of linoleic acid and larger proportion of saturated acids than cardiolipin.

(*d*) Polar lipid fraction. Paper chromatography showed a weak lipid spot, whose R_F corresponded roughly to that of sphingomyelin. It was choline-negative but the amount of choline may have been

below limits of detection. A ninhydrin-positive spot remained at the origin.

The composition of polar fractions is given in Table 18. The fraction gave a very high yield of methyl esters after sublimation (cf. Table 4: homogenate Fractions 8 and 9, mitochondria Fraction 8, microsomes Fraction 8).

The lipids present in this minor fraction have not been characterized. Their fatty acid distribution is shown in Table 19. The detection of only a small number of fatty acids in polar lipids of mitochondria and microsomes is probably due to the insufficient amount of material available for gas chromatography.

(*e*) Neutral lipids. Paper chromatography showed spots corresponding to mono-, di- and triglycerides, cholesterol and cholesterol esters, possibly free fatty acids and a lipid spot in Fraction 1 (Table 1) moving with the solvent front.

The phosphorus content shows that phospholipid contamination is small (Table 20). The analyses (except of cholesterol) were done at the lower limits of their sensitivity and are only approximate. The low recovery of cholesterol is discussed in the 'Evaluation of Methods' section (Lipid fractionation).

Table 21 shows the fatty acid distribution in neutral-lipid fractions. Neutral-lipid fractions are remarkable for the large variety of fatty acids present in them. They also contain high proportions of compounds C and D.

As may be seen from Table 5, on the non-polar stationary phase compound C co-chromatographed with myristate and compound D with palmitate. The compounds ran much slower on polar stationary phases: C in front of 20–1 and D in front of arachidonate. The change in retention volumes between the two types of stationary phase is larger than would be expected to result from unsaturation of the fatty acid chain and suggests the presence of a more polar group.

The compounds constitute 70 % of mitochondrial and 30 % of microsomal neutral lipids. Their proportion in homogenate neutral lipids is uncertain, since these lipids were chromatographed only on the non-polar stationary phase.

Trace amounts of compound C were also detected in some kephalin and polar lipid fractions.

Table 19. *Fatty acid distribution in polar lipid fractions*

Chain $n-x$ contains n C atoms and x double bonds. un., Unsaturated; br., branched.

$n-x$	Description or trivial name	Methyl ester in measurable peaks (%)		
		Homogenate	Mitochondria	Microsomes
	Esters			
14–0	Myristic	1·9	–	4·4
14–1	.	+	–	1·9
15–0	.	–	–	2·7
15–1	.	+	–	1·6
16–0	Palmitic	34·5	32·2	25·8
16–1	Palmitoleic	3·1	–	12·7
17–br.	.	–	–	1·4
18–0	Stearic	25·0	40·2	24·1
18–1	Oleic	30·3	23·3	24·5
18–2	Linoleic	+	+	1·0
20–4	Arachidonic	4·5	–	–
22–5	.	–	4·3	–
	Trace components (esters)			
8–0	.	–	–	+
10–0	.	–	–	+
12–0	Lauric	–	–	+
13–0	.	–	–	+
13–un.	.	–	–	+
20–0	Arachidic	–	–	+
20–1	.	+	–	+
C	Unidentified highly polar component	–	–	+
	Summation of esters			
$n-0$	.	61·4	72·4	57·0
$n-1$	.	33·4	23·3	38·8
$n-2$	.	+	+	1·0
$n-x>2$	.	4·5	4·3	–
	Aldehyde			
H	See Table 5 .	0·8	–	2·7
A	See Table 5 .	–	–	1·6
B	See Table 5 .	–	–	2·1

Table 20. *Composition of neutral-lipid fractions*

	Composition (moles/100 moles of methyl ester)		
	Homogenate	Mitochondria	Microsomes
Total P (≡ phospholipid contamination)	0·47	4·6	12
Amino N	6·0	0·3	1·8
Total N	.	20	.
Cholesterol recovered in fraction	148	33	95

Table 21. *Fatty acid distribution in neutral-lipid fractions*

Chain $n-x$ contains n C atoms, x double bonds. un., Unsaturated; br., branched.

$n-x$	Description or trivial name	Methyl ester in measurable peaks (%)		
		Homogenate	Mitochondria	Microsomes
	Esters			
8–0	.	–	–	2·6
12–0	Lauric	–	0·7	+
12– or 13–un.	.	–	–	2·3
13–0	.	0·7	+	+
14–0	Myristic	0·9*	0·3	+
14–1	.	+	0·3	+
15–0	.	0·2	0·3	0·4
15–1	.	0·4	+	0·4
16–0	Palmitic	29·0*	6·7	14·5
16–1	Palmitoleic	4·5	1·0	1·2
17–br.	.	0·3	+	+
18–0	Stearic	26·6	2·5	22·2
18–1	Oleic	23·9	2·6	11·9
18–2	Linoleic	1·3	1·3	–
20–1	.	+	1·8	0·7
20–4	Arachidonic	9·1	–	8·0
22–5	.	–	5·2	–
22–6	.	1·4	–	2·0
C, D	Unidentified polar component	*	17·8	7·2
		*	59·6	26·6
	Trace components (esters)			
10–0	.	–	–	+
13–un.	.	–	–	+
14–1	.	–	–	+
20–0	.	–	–	+
F	Unidentified	–	–	+
K	Unidentified	–	+	–
H, A, B	See Table 5 — Aldehydes			
H	.	0·9	0·3	4·0
A	.	0·8	1·1	–
B	.	+	0·2	2·1

* Figures for homogenate acids 14–0 and 16–0 may include C and D. No separation was possible as samples were chromatographed only on non-polar stationary phase (see Results section: 'Neutral lipids').

DISCUSSION

Similarity of mitochondrial and microsomal lipids

Mitochondrial, microsomal and total-brain phospholipids contain similar proportions of the two major components, lecithin and kephalin (phosphatidylserine and phosphatidylethanolamine and corresponding plasmalogens). The fatty acid composition of either of these lipid classes shows only minor variations from one centrifugal fraction to another. These findings are similar to those of Macfarlane *et al.* (1960) for rat liver and Marinetti *et al.* (1958) for pig heart.

The similarity of mitochondrial and microsomal phospholipids despite the diverse functions of these organelles suggests that a large proportion of the phospholipid in cell organelles has no direct metabolic function, but serves as a structural component common to mitochondria and microsomes. The differences in the distribution of fatty acids in the same phospholipid class in mitochondria and microsomes may represent the content of a small proportion of the lipid with a distinctive fatty acid composition, derived from specific structures more directly involved in metabolism than the bulk of the lipid. Similar con-

siderations may apply to minor phospholipid components (polyglycerophosphatides, phosphoinositides, sphingomyelin) on which the present study throws little light.

Two lines of approach may lead to the identification of lipids with specific metabolic functions. One is the study of lipids in subfractions of mitochondria and microsomes (Green, 1959; Siekievitz, 1959). Some of these particles may contain a metabolically important lipid in higher concentration than total cell subfractions (cf. Holman & Widmer, 1959). The other, parallel, approach is more exact characterization of the tissue lipids by improvements in methods of fractionation and analysis.

Specific composition of brain-lipid classes

The fatty acid composition of each brain phospholipid is specific both in proportions of fatty acids with different numbers of double bonds and in relative proportions of fatty acids with the same degree of saturation (cf. differences in palmitate/stearate ratio between lecithin and kephalin). The differences in fatty acid distribution between brain lecithin and kephalin bear no close relationship to the differences between corresponding lipid fractions in rat liver.

This specificity indicates that the fatty acid structure of a particular phospholipid is not simply a function of total fatty acid distribution in the organ. It is possible that a mechanism of diglyceride synthesis exists which is specific for each lipid in the selection of fatty acids esterified as well as (at least for lecithin) in the position of the saturated fatty acids on glycerol (cf. Tattrie,1960; Hanahan, Brockerhoff & Barron, 1960).

Fatty acid distribution in total-brain phospholipids

All classes of brain phospholipids analysed contained considerably more saturated fatty acids (40–60%) and much less polyethenoid fatty acids (10–25%) than has been reported for rat liver (Dittmer & Hanahan, 1959; Macfarlane *et al.* 1960) or ox-heart mitochondria (Holman & Widmer, 1959). A polyglycerophosphatide fraction rich in linoleic acid which has been isolated from rat-liver mitochondria (Getz & Bartley, 1959) and ox heart (Macfarlane *et al.* 1960) is either absent from brain or present to the extent of below 2% of the lipid phosphorus in mitochondria and microsomes.

In view of these findings it seems that in brain mitochondria a high total content of polyethenoid fatty acids in the constituent phospholipids is not essential for the functioning of respiratory enzyme systems.

SUMMARY

1. Lipids from rat-brain homogenate, mitochondrial and microsomal fractions were analysed for phosphorus, total nitrogen, amino nitrogen, choline, cholesterol, acyl ester and aldehyde content. They were separated into lipid classes by silicic acid-column chromatography. The distribution of fatty acids in whole lipids and the lipid classes was obtained by gas-chromatographic analysis of methyl esters of the fatty acids.

2. Lipids from all three tissue fractions were similar in their content of different lipid classes and in the fatty acid distribution both in total-lipid extracts and in individual lipid classes. Proteolipids resembled total-lipid extracts in their fatty acid distribution.

3. In comparison with other lipids, brain phospholipids are remarkable for their high content of saturated fatty acids (50%) and low proportion of essential (polyenoic) fatty acids (16%). Linoleic acid accounts for only 1% of total fatty acids.

4. Mitochondria contained 26% of their tissue dry weight as phospholipid, 6% as cholesterol and 4% as neutral fat.

5. The composition of mitochondrial phospholipid was 32% of lecithin, 50% of phosphatidylserine and phosphatidylethanolamine, 13% of phosphoinositide, 2% of polyglycerophosphatide, 17% of plasmalogens (serine and ethanolamine). No lecithin plasmalogens were found.

6. Fatty acids from total mitochondrial extract contained 28% of palmitic acid, 23% of stearic acid, 32% of oleic acid, 11% of essential acids (linoleic acid and polyenoic acids) and traces of short-chain acids ($C_{n<16}$).

7. Mitochondrial serine–ethanolamine phosphatide fraction contained 9% of palmitic acid, 32% of stearic acid, 30% of oleic acid and 23% of essential fatty acids. Mitochondrial lecithin fatty acids consisted of 39% of palmitic acid, 17% of stearic acid, 32% of oleic acid and 8% of essential fatty acids. Polyglycerophosphatide fraction contained 13% of palmitic acid, 21% of stearic acid, 19% of oleic acid, 25% of essential acids and 4% of short-chain acids.

8. No close resemblance was found between the composition of any brain-lipid class and similar lipids from other organs, but some general trends were apparent. The findings are discussed in connexion with possible metabolic functions of lipids.

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Studies on Cerebral Sphingomyelin

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Sphingomyelin was first isolated from brain by Thudichum (1884) but little was known of its metabolism until recently. Brady & Koval (1958) and Brady, Formica & Koval (1958) have now carried out extensive investigations on sphingosine biosynthesis, and more recently Sribney & Kennedy (1958) have shown that the biosynthesis of sphingomyelin in liver proceeds by the reaction of a ceramide (*N*-acylsphingosine) with cytidine diphosphate choline. It is quite likely that such a reaction proceeds in nervous tissue (Rossiter, Thompson & Strickland, 1960).

In tissues other than brain the function of sphingomyelin is unknown. In nervous tissue the lipid probably does not make its appearance until the onset of myelination when it rapidly increases in concentration (Folch-Pi, 1955). Earlier investigations (Branté, 1949; Johnson, McNabb & Rossiter, 1949) have shown that the sphingomyelin is preferentially located in the white matter (myelinated fibres), and Finean and his colleagues have suggested that the lipid could fit well into the structure of the repeating units of myelin (Finean & Robertson, 1958). In this sense therefore, white-matter sphingomyelin would appear to have a structural role in the nervous system, although this may not be so for sphingomyelin occurring outside the myelin sheath, e.g. in cerebral cortex. From experiments with [3-^{14}C]serine, Davison, Morgan Wajda & Payling-Wright (1959) have concluded that the sphingomyelin in myelin has a very slow rate of replacement and may even be permanent

when laid down in early life. Indeed, earlier experiments by Dawson (1954*a*) strongly suggested that *in vivo* the uptake of ^{32}P into guinea-pig-brain sphingomyelin was very slight when compared with that of other phospholipids.

The findings of Rennkamp (1949) and Dawson (1958) showed that sphingomyelin can be hydrolysed to yield a mixture of phosphorylcholine and sphingosylphosphorylcholine which can subsequently be isolated. This provides a convenient method for measuring the uptake of isotopic phosphorus into sphingomyelin in small samples of tissue and this paper deals in part with the measurement of the uptake of ^{32}P into rat-brain sphingomyelin *in vivo*. The use of the method to estimate true brain sphingomyelin has also been investigated (cf. Dawson, 1960). A preliminary account of these studies has been given by Ansell & Spanner (1959).

EXPERIMENTAL

Preparation of lipid extracts. These were prepared in two ways. In the majority of experiments with ^{32}P the fresh tissue was homogenized in a mechanical blender with 12% trichloroacetic acid, washed and suspended in acetone, and the residue extracted with $CHCl_3$–methanol (1:1, v/v) as described by Ansell & Dohmen (1956). In other experiments tissue was homogenized in $CHCl_3$–methanol (2:1, v/v), the homogenate filtered and the filtrate dried three times *in vacuo* (Davison & Wajda, 1959). The residue was taken up in $CHCl_3$–methanol (2:1, v/v) and filtered. It was then washed with 0·2 vol. of 0·73% NaCl followed by chloroform–methanol–0·29% NaCl (3:48:47, by vol.) as described by Folch, Lees & Sloane-Stanley (1957), and the lipid solution diluted to standard volume. Lipid solutions prepared in this way were virtually colourless.

Saponification of lipids. Saponification was carried out essentially as described by Schmidt, Benotti, Hershman & Thannhauser (1946). A suitable sample of the lipid solution (e.g. an amount equivalent to 400 mg. of brain tissue) was dried *in vacuo* at $< 40°$ and emulsified with 5 ml. of N-NaOH. After 2–16 hr. at 37° the emulsion was cooled and neutralized with 5N-HCl, and 5·9 ml. of 10% trichloroacetic acid added. After 2 hr. at 4° the suspension was filtered and the residue on the paper washed with 2% trichloroacetic acid until the filtrate was free from chloride. The residue on the paper (lipid stable to dilute alkali and acid) was then dissolved in warm $CHCl_3$–methanol (1:1, v/v). Alternatively the acidified suspension of lipid after saponification was shaken with an equal volume of warm $CHCl_3$–methanol (1:1, v/v), and the $CHCl_3$ layer which was obtained on centrifuging washed by shaking with methanol–water until free from chloride.

Hydrolysis of mild acid- and alkali-stable fraction. The solution of this fraction was then dried under reduced pressure at $< 40°$ and dissolved in 3 ml. of methanolic 2N-HCl. It was then heated at 100–110° in a sealed tube for 1·5 hr. (Dawson, 1958). By adding 3 ml. of $CHCl_3$ and 3 ml. of water and centrifuging, $CHCl_3$-soluble and water-soluble phosphorus fractions were obtained.

Chromatographic separation of water-soluble hydrolysis products. The aqueous phase after hydrolysis of acid- and alkali-stable lipid in 2N-HCl was dried under reduced pressure and stored overnight *in vacuo* over NaOH to remove the last traces of HCl. Single-dimensional chromatography in phenol–water–aq. 18N-NH_3 soln. (80 g.: 20 ml.:0·3 ml.) was carried out on Whatman no. 1 paper (previously freed from cations by washing with 2N-acetic acid and glass-distilled water). On some occasions sphingosylphosphorylcholine was separated from phosphorylcholine in butan-1-ol–acetic acid–water (Dawson, 1958). Phosphorus compounds were generally located by spraying dried papers with the reagent of Hanes & Isherwood (1949), drying at room temperature and exposing to the unfiltered light from a Hanovia 100w mercury-vapour lamp. The method of Wade & Morgan (1953) was also used when it was desired to elute phosphate esters unchanged from the paper. Amino compounds were detected by spraying with 0·5% ninhydrin in water-saturated butanol, drying at room temperature and then heating the papers at 100° for 2–5 min. The method of Levine & Chargaff (1951) was used to detect choline compounds after destruction of ninhydrin-reacting material (Lea, Rhodes & Stoll, 1955). Radioautographs of chromatograms were prepared with Kodak 'Blue Brand' X-ray film.

Preparation of sphingomyelin. For a large part of the work a sample of sphingomyelin prepared from brain by the method of Klenk & Rennkamp (1941), and kindly donated by Dr J. N. Hawthorne, was used. This contained a small amount of water ($< 5\%$) and had a P content of 3·6% and an N content of 3·1% (N:P ratio, 1·9:1·0). In further experiments sphingomyelin in small amounts was prepared as follows (cf. Davison & Wajda, 1959). Ether-insoluble lipids (500 mg.) from ox brain, kindly donated by Dr P. Kemp, were dissolved in $CHCl_3$–methanol (98:2, v/v), run through a column containing 50 g. of Al_2O_3 (British Drug Houses Ltd. chromatographic, as purchased) and washed through with a further 50 ml. of solvent. All the P was retained. The column was then eluted with 500–600 ml. of $CHCl_3$–methanol (1:1, v/v). According to Davison & Wajda (1959) after C. Long (unpublished work) this elutes choline-containing lipids but no cerebroside or 'kephalin' components. Chromatography of this fraction on silica-impregnated paper in di-*n*-butyl ether–acetic acid–$CHCl_3$–water (40:35:6:5, by vol.) (Marinetti & Stotz, 1956) showed the presence of at least two choline-containing lipids. Accordingly the eluate from the column was dried under reduced pressure and saponified for 8 hr. at 37° in methanolic N-NaOH. The solution was made just acid with HCl and the lipid extracted into $CHCl_3$ by shaking with an equal volume of $CHCl_3$–methanol (1:1, v/v). Then, freed from chloride and water-soluble alkali-labile phosphate by repeated washing with methanol–water, the $CHCl_3$ layer was dried and the residue dissolved in about 10 ml. of light petroleum (b.p. 60–80°)–methanol (9:1, v/v). Phospholipid was precipitated from this solution with acetone, washed with this solvent and dried *in vacuo*. The lipid was then dissolved in the minimum of hot ethyl acetate containing about 5% (v/v) of methanol. On cooling, a white solid crystallized; this was then recrystallized and dried *in vacuo*. From 460 mg. of starting material containing 9·4 mg. of P an amount of lipid equivalent to 2·8 mg. of P was obtained. It was slightly hygroscopic: H_2O content, 6·18% after drying at 100° for 3 hr. *in vacuo* (Found for dried sample: C, 68·67; H, 11·51; N, 3·63;

P, 4·05%; N:P ratio, 1·99:1. Calc. for *N*-stearylsphingomyelin $C_{41}H_{85}O_7N_2P$: C, 65·71; H, 11·44; N, 3·74; P, 4·15%. Calc. for *N*-nervonylsphingomyelin $C_{47}H_{95}O_7N_2P$: C, 67·88; H, 11·53; N, 3·37; P, 3·74%). Elementary analyses were carried out by Dr F. Pascher, Bonn, Buschstrasse 54.

No carbohydrate contaminant was detected by the Molisch reaction. The lipid migrated as a single substance in di*iso*butyl ketone–acetic acid–water (40:30:7, by vol.) (Marinetti & Stotz, 1956) on silica-impregnated paper and gave no reaction with ninhydrin. On hydrolysis with methanolic HCl and subsequent separation of water-soluble P compounds by paper chromatography, 85% of the P present could be recovered as a mixture of phosphorylcholine and sphingosylphosphorylcholine. In all respects this lipid behaved essentially the same as the sample prepared by the method of Klenk & Rennkamp (1941). Determination of the infrared spectra of the two samples by Dr K. J. Morgan showed that the two preparations were indistinguishable. The method described here would appear to provide a convenient method of preparing small amounts of sphingomyelin if the starting material used by Rapport & Lerner (1958) is unavailable. A sphingomyelin has recently been totally synthesized by Shapiro, Flowers & Spector-Shefer (1959).

Phosphorus and nitrogen determinations. P was determined either by the method of Fiske & Subbarow (1925) or, when the amounts were between 1 and 5 μg., by the method of Berenblum & Chain (1938). Spots on chromatograms and appropriate blanks were wet-ashed with $HClO_4$ before P determination. N determinations were carried out by the Conway method as modified by Weil-Malherbe & Green (1955); this method was applied to both lipid samples and aqueous eluates of phosphate esters from chromatograms, sprayed with the $FeCl_3$–sulphosalicylic reagent of Wade & Morgan (1953).

Radioactive assay. This was carried out on aqueous solutions of ^{32}P in an M6 liquid counting tube (20th Century Electronics) with conventional ancillary equipment.

RESULTS

Acid hydrolysis of sphingomyelin

In agreement with the observations of Dawson (1958), the hydrolysis of sphingomyelin with methanolic 2N-hydrochloric acid for 1·5 hr. at 100° in a sealed tube yields two water-soluble phosphate esters which migrate to the same position (R_p 0·95) in phenol–ammonia but which can be separated in butan-1-ol–acetic acid–water. With anhydrous methanol the yield of sphingosylphosphorylcholine was small when compared with the yield of phosphorylcholine, but the amounts could be made equal by the presence of 20% (v/v) water in the methanolic acid (Fig. 1). In this experiment the recovery of phosphorus as a mixture of esters was only about 75% of the total phosphorus in the sphingomyelin, though no phosphorus was detected in the chloroform layer after hydrolysis of the sphingomyelin and extracting with this solvent. The relative proportions of the esters were not affected by increasing the time of hydrolysis at 100–110°, nor was the total yield of water-soluble phosphorus increased.

Acid hydrolysis of acid- and alkali-stable fraction of brain lipids

Although the acid hydrolysis of pure sphingomyelin yielded the greater part of its phosphorus as a mixture of sphingosylphosphorylcholine and phosphorylcholine, this was not true for the alkali-stable fraction of rat-brain lipids. This difference was indeed anticipated because the fraction is known to contain phospholipids other than sphingomyelin (Branté, 1949; Dawson, 1954*b*). When the water-soluble fraction after acid hydrolysis was subjected to single-dimensional chromatography in phenol–ammonia a further phosphorus compound (possibly two) was found with an R_p of about 0·2 (X in Table 1). Further, a considerable amount of phosphorus was found in the chloroform layer after the extraction procedure described in Methods. When methanolic hydrochloric acid containing 20% of water was used there seemed little doubt, to judge from recovery experiments, that a proportion of this chloroform-soluble phosphorus was unchanged sphingomyelin. However, when anhydrous methanolic hydrochloric acid was used, recovery of added sphingomyelin as water-soluble ester phosphorus was good (Table 1), and it was considered that the chloroform-soluble phosphorus must represent some strongly bound lipid phosphorus.

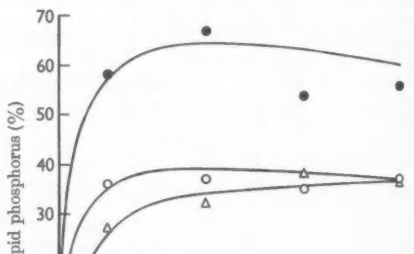

Fig. 1. Hydrolysis of sphingomyelin by hydrochloric acid at 100–110°. (*a*) 2N-HCl in anhydrous methanol: ●, phosphorylcholine; ▲, sphingosylphosphorylcholine. (*b*) 2N-HCl in methanol–water (80:20, v/v): ○, phosphorylcholine; △, sphingosylphosphorylcholine.

Table 1. *Recovery of sphingomyelin phosphorus added to brain-lipid extracts prepared from young rats*

The fractions were separated as described in the text. Values are given as μg. of P/g. of brain

		X	$CHCl_3$ layer	Sphingosyl-phosphorylcholine–phosphorylcholine	Recovery (%)
Brain lipid ≡ half brain	(*a*)	21·6	26·2	29·6	—
	(*b*)	18·0	23·2	29·2	—
	(*c*)	17·4	24·4	28·8	—
Brain lipid ≡ half brain +29·9 μg. of sphingomyelin P	(*a'*)	17·2	26·2	56·0	88
	(*b'*)	20·0	—	57·4	94
	(*c'*)	16·8	24·8	56·0	91

Table 2. *Effect of chloroform extraction on the amount of sphingosylphosphorylcholine–phosphorylcholine found in the acid hydrolysate of the alkali-stable phospholipid fraction (guinea-pig brain)*

The methanolic-HCl hydrolysate was divided into two parts (*a*) and (*b*). An aqueous suspension of (*a*) was subjected to chromatography in its entirety and (*b*) was extracted with a mixture of chloroform and water to yield two phases; each was separately subjected to chromatography. Values are given as μg. of P/g. of brain.

	X	Sphingosylphosphorylcholine–phosphorylcholine
(*a*) Total hydrolysate	41	174
(*b*) Chloroform phase	None	61
Water phase	41	100
(*a*) Total hydrolysate	49	187
(*b*) Chloroform phase	None	37
Water phase	49	112

However, in the experiments of Dawson (1960 and personal communication) in which the total acid hydrolysate of the acid- and alkali-stable fraction was subjected to chromatography without prior extraction with chloroform, all the phosphorus could be accounted for as 'sphingosylphosphorylcholine–phosphorylcholine' and other water-soluble esters. It was observed from our own experiments that the phosphorus in the chloroform-soluble fraction, when added to the sphingosylphosphorylcholine–phosphorylcholine phosphorus, gave values for guinea-pig-brain sphingomyelin that agreed with those of Dawson (1954*b*) based on total choline in the acid- and alkali-stable fraction (Table 2). Further, when the chloroform-soluble phosphorus was subjected to chromatography either in phenol–ammonia or in butan-1-ol–acetic acid, there was one phosphorus spot which ran to the position of sphingosylphosphorylcholine and reacted with ninhydrin. Subjection of the total hydrolysate to chromatography resulted in a much greater amount of 'sphingosylphosphorylcholine–phosphorylcholine' and no other substances (Table 2) although the chromatogram was rather distorted. Hence the chloroform-soluble phosphorus was behaving in many respects in these experiments like the water-soluble sphingosylphosphorylcholine.

When a sample of the chloroform-soluble fraction from guinea-pig brain was dried and heated in a sealed tube with aqueous 2N-hydrochloric acid at 123° for 46 hr., the phosphorus was rendered water-soluble as inorganic phosphorus. The water-soluble products of this drastic hydrolysis were then subjected to chromatography on potassium chloride-impregnated papers in the butan-1-ol–phenol–formic acid–water system of Bremer & Greenberg (1959). Two ninhydrin-reacting substances were obtained, one of which had an R_F identical with ethanolamine hydrochloride. Choline, which in this system is well separated from ethanolamine, mono- and di-methylethanolamine, could not be detected by the Dragendorff reaction, nor could choline be detected in the hydrolysate by the conventional reineckate method.

From these observations it was concluded that the chloroform-soluble phosphorus obtained on methanolic hydrochloric acid hydrolysis of the alkali-stable brain-phospholipid fraction was not a form of sphingosylphosphorylcholine. It seems unlikely that a phospholipid containing acyl groups would survive saponification and methanolic hydrochloric acid, but attempts to prove this were foiled by the presence of large amounts of methyl esters of fatty acids as contaminants. It is possible that the lipid may be comparable with that recently described by Svennerholm & Thorin (1960).

In a personal communication, Dr R. M. C. Dawson has indicated that he is now of the opinion that his sphingosylphosphorylcholine–phosphorylcholine fraction (Dawson, 1960) may well contain another component.

Estimation of true sphingomyelin in brain

Most estimations of tissue sphingomyelin have been carried out by determining the alkali-stable phosphorus by the method of Schmidt *et al.* (1946) and this has been so for many estimations on nervous tissue (e.g. Johnson *et al.* 1949). True sphingomyelin or mild-alkali-stable choline-containing phospholipid has been determined by prolonged hot alkaline hydrolysis of the alkali-stable

fraction to liberate choline (Branté, 1949; Dawson, 1954*b*), which can be subsequently estimated.

There are very few published values for the true sphingomyelin content of brain tissue. Branté (1949) gave some values for young rats based on the choline content of the acid- and alkali-stable phospholipid fraction, and Dawson (1954*b*) has given values for the true sphingomyelin content of adult guinea-pig brain (170 μg. of P/g. of tissue), whereas calculation from the results of Edgar & Smits (1959) shows that adult rabbit telencephalon contains 172 μg. of P/g. as sphingomyelin. Dawson (1960) has estimated the sphingomyelin content of sheep brain to be 207 μg. of P/g. of tissue. The application of the present modification of the Dawson (1958) method has been applied to the determination of sphingomyelin in the brains of rats of different ages after cold extraction with chloroform–methanol, and the results are shown in Table 3, which also lists total lipid phosphorus, acid- and alkali-stable phosphorus and the contributions from the unknown phospholipids. In Table 4 are given values for ox grey and white matter for comparison with the values of Branté (1949).

Nature of the unknown water-soluble phosphate components of the acid- and alkali-stable fraction

The nature of the water-soluble phosphorus-containing acid-hydrolysis products of the alkali-stable fraction (X in the tables) is unknown. The amount obtained was variable and sometimes two distinct spots in addition to phosphorylcholine–sphingosylphosphorylcholine could be seen in the single-dimensional phenol–ammonia chromatograms; more usually there was only one. Two-dimensional chromatography in ethanol–13N-ammonia solution (60:40, v/v) and phenol–ammonia did not resolve the predominant spot further, but in butan-1-ol–acetic acid–water (12:3:5, by vol.) (upper phase) two smaller spots were often seen in addition to the larger one. These additional phosphorus-containing areas did not react with ninhydrin, though the combined areas from a single-dimensional chromatogram contained a large amount of total nitrogen relative to the phosphorus present. Nitrogen:phosphorus ratios varied considerably, however, from 2·3 to 8·5, and it seemed possible that the nitrogen component ran coincidentally with the phosphorus-

Table 3. *Amount of total lipid phosphorus, acid- and alkali-stable lipid phosphorus and sphingomyelin phosphorus in the whole brains of rats of increasing age*

Values are given as μg. of P/g. of fresh brain tissue.

Age	Total lipid	Total acid- and alkali-stable lipid	Sphingomyelin (water-soluble sphingosylphosphoryl-choline–phosphoryl-choline)	Chloroform-soluble P	X
8 days	760–800 (2)	50–52 (2)	8–9 (2)	15 (2)	9–13 (2)
2 weeks	922–1005 (3)	59–92 (3)	13–16 (3)	19 (3)	6–32 (3)
3–4 weeks	1443±19* (13)	143±1·7* (13)	45±3* (13)	25–27 (3)	28 (3)
5–6 weeks	1460–1511 (2)	143–194 (2)	54–56 (2)	42–44 (2)	24–44 (2)
8–9 weeks	1485–1500 (2)	158–165 (2)	56 (2)	46–47 (2)	27–28 (2)
9–11 weeks	1661±183* (6)	181±18* (6)	56·0±9·3* (6)	44±7* (6)	35±12* (6)
52 weeks	1655–1800 (2)	192–200 (2)	67–72 (2)	40–41 (2)	30–50 (2)

* S.D.

Table 4. *Total alkali-stable phospholipid and sphingomyelin of grey and white matter of the brain*

Values are given as μg. of P/g. of tissue.

		Alkali-stable phospholipid	Sphingomyelin	Chloroform-soluble fraction	X
Present paper					
Ox	grey (cortex)	160	23*	60	38
	white (corpus callosum)	665	358*	99	107
Calculation from Branté (1949)					
Ox	grey	—	164†	—	—
	white	—	480±120†	—	—
Man	grey	156–300	108±68†	—	—
	white	—	432±152†	—	—

* Calculated from water-soluble sphingosylphosphorylcholine–phosphorylcholine.
† Calculated from choline estimation.

Table 5. *Effect of trichloroacetic acid precipitation on the recovery of rat-brain phospholipid fractions*

Values are given as μg. of P/g. of tissue.

Age (weeks)	Treatment*	Total lipid	Acid- and alkali-stable lipid	X	Sphingomyelin (water-soluble phosphoryl-choline–sphingo-sylphosphoryl-choline)	Chloroform-soluble P
9–11	Trichloroacetic acid	1330	118	8	43	37
	Direct extraction	1461	170	30	41	34
9–11	Trichloroacetic acid	1405	128	8	43	43
	Direct extraction	1448	184	27	54	45
>11	Trichloroacetic acid	1520	163	21	54	45
	Direct extraction	1720	206	58	64	54
>11	Trichloroacetic acid	1570	153	20	56	51
	Direct extraction	1680	196	38	68	47

* See text.

containing component in phenol–ammonia. There was a considerable decrease in the amount of this unknown phosphorus fraction when the tissue was treated first with trichloroacetic acid, indicating that the component(s) is partially acid-labile. This may be linked with the fact that treatment of brain tissue with trichloroacetic acid decreased the amount of total lipid phosphorus and acid- and alkali-stable lipid phosphorus obtained on subsequent extraction with solvents, in comparison with that obtained by direct solvent extraction of the tissue (see Experimental section) (Table 5). This effect of trichloroacetic acid confirms observations made by Dawson (1954*b*) and Robins, Lowry, Eydt & McCaman (1956). The total decrease in acid- and alkali-stable lipid phosphorus was by no means accounted for by the decrease in X phosphorus, although the amount of sphingomyelin recovered was much less affected by the treatment with trichloroacetic acid. Whichever method was used the sphingomyelin phosphorus, the chloroform-soluble phosphorus and X phosphorus never accounted for the whole of the acid- and alkali-stable phosphorus.

Attempts to separate the phospholipid components of the acid- and alkali-stable fraction by first separating them from fatty acids with silicic acid (Borgström, 1952) and then subjecting a methanol eluate of the phospholipid material to chromatography on silicic acid-treated papers were unsuccessful.

Uptake of [^{32}P]*orthophosphate into brain sphingomyelin*

When [^{32}P]orthophosphate was injected intraperitoneally into 3- to 4-week-old rats there was a steady uptake into both phosphatidylcholine and sphingomyelin of the brain. Estimation of the specific radioactivity of phosphatidylcholine was by the method of Dawson (1954*c*), and, as discussed elsewhere (Ansell & Spanner, 1960), the incorporation into this lipid in brain followed a different course from that in liver. Fig. 2 shows that young rat brain (now past the stage of maximum rate of myelination which reaches its peak at 14–21 days after birth) incorporated phosphate at a rate approximately half that of phosphatidyl-

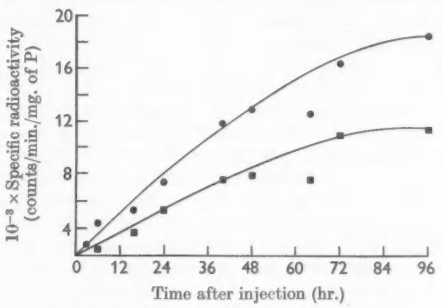

Fig. 2. Uptake of ^{32}P into the cerebral phosphatidylcholine (●) and sphingomyelin (■) of young rats.

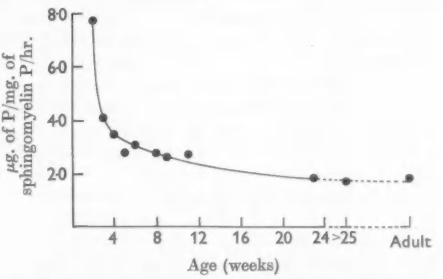

Fig. 3. Exchange of phosphorus in the cerebral sphingomyelin of rats of increasing age.

choline. This rate must represent a mean for all the brain sphingomyelin wherever it is situated. As discussed below, however, the difficulties of interpretation are ubiquitous.

An attempt is made in Fig. 3 to demonstrate the variation in the rate of uptake with increasing age of the rat. Rats were injected with ^{32}P and killed after 24 hr., over which period the uptake of isotope into sphingomyelin is largely linear (Fig. 2), and the ratio of the specific radioactivity of the sphingomyelin phosphorus to that of the acid-soluble phosphorus was used as an arbitrary measure of phosphate exchange (Dawson & Richter, 1950; Ansell & Dohmen, 1957). This value was calculated as μg. of P incorporated/μg. of sphingomyelin P/hr. and it is apparent that there was a considerably greater rate of incorporation per μg. of P in the young rat than in the old, although, as the amount of sphingomyelin in the brain of the young rat is much smaller (Table 3), it might be inferred that the rates of synthesis of sphingomyelin per gram of brain are not dissimilar.

Although it is difficult to obtain pure 'white matter' from rat brain (Majno & Karnovsky, 1957), an attempt was made to compare the uptake of ^{32}P into the sphingomyelin of grey and white matter in the adult rat brain. Adult rats (about 1 year old) were injected with [^{32}P]orthophosphate (4 μc/g. body weight) and the brains removed after 24 hr. The corpus callosum and optic chiasma of each were pooled to give a total yield of about 10 mg. of white matter for each rat. About 200 mg. of cortical grey matter was also obtained from each brain and the specific radioactivity of the sphingomyelin in each sample determined. There was a definite sphingosylphosphorylcholine–phosphorylcholine spot from grey matter confirming that sphingomyelin occurs outside myelin (cf. Robins, Eydt & Smith, 1956; Edgar & Smits, 1959), and this was significantly radioactive, whereas the spot from white matter was negligibly labelled (Table 6). It would appear from these results that cortical sphingomyelin has a turnover in adult life not shared by its counterpart in the white matter.

Table 6. *Uptake of* [^{32}P]*orthophosphate into the grey- and white-matter sphingomyelin of the adult rat*

The animals received 4 μc of [^{32}P]orthophosphate/g. of body weight and were killed 24 hr. after the injection.

	Specific radioactivity (counts/min./mg. of P) of sphingomyelin	
Rat no.	Cortical grey tissue	Corpus callosum
1	19 500	< 3770
2	18 400	< 1940

DISCUSSION

The present experiments indicate that sphingomyelin represents only a portion of the phospholipid obtained after mild acid and alkaline treatment of brain phospholipids, confirming the earlier observations of Brante (1949) and Dawson (1954*b*) among others. In the whole brain of adult rats, sphingomyelin phosphorus only accounts for about one-third of the phosphorus stable to mild acid and alkali treatment, whereas for white matter from ox corpus callosum it accounts for 70%. The remaining phosphorus in our experiments gave rise to unidentified water-soluble hydrolysis products and a chloroform-soluble fraction, which did not, however, account for all the phosphorus in the alkali-stable fraction. According to Dawson (1960) sphingomyelin accounts for about 60% of the phospholipid in whole sheep brain which is stable to mild acid and alkali.

Precipitation of brain tissue with trichloroacetic acid before extraction with lipid solvents only slightly affected the recovery of sphingomyelin, but it had an adverse effect on the unknown phospholipid (X) the amount of which was considerably decreased (Table 5). Dawson (1954*b*) also observed this effect of acid on the unknown alkali-stable phospholipid(s). Edgar & Smits (1959) noted that after freeze-drying of the tissue lower values were obtained for this fraction than after direct extraction of the wet tissue. The very recent experiments of Dawson (1960) indicate that direct extraction is the method of choice for the total analysis of tissue phospholipids. The fact that treatment of the tissue with cold trichloroacetic acid reduced the amount of the predominant unknown water-soluble phosphate ester obtained suggests that it may derive from a phospholipid containing an acid-sensitive linkage, e.g. an unsaturated ether as found in plasmalogens.

It is apparent, however, that until the nature of this mild acid- and alkali-stable lipid(s) is known the factors which affect its recovery from tissue samples during an analytical procedure are likely to remain obscure. Carter, Smith & Jones (1958) isolated an acid- and alkali-stable phospholipid from egg yolk which was shown to consist largely of a phosphorylethanolamine derivative of batyl alcohol and they suggested that such a lipid may be present in brain tissue. Very recently Svennerholm & Thorin (1960) have obtained more definite evidence of an acid- and alkali-stable 'kephalin B' from brain which yielded ethanolamine on strong acid hydrolysis and was comparable analytically with the product of Carter *et al.* (1958). In the present work no apparent water-soluble ninhydrin-positive material apart from sphingosylphosphorylcholine was obtained on methanolic hydro-

chloric acid hydrolysis of the acid- and alkali-stable phospholipids of brain, and more than one water-soluble phosphate ester was often obtained in addition to those derived from sphingomyelin. However, it seems possible from our experiments that the chloroform-soluble phosphorus remaining after methanolic hydrochloric acid hydrolysis, which was shown to react with ninhydrin and to yield a component provisionally identified as ethanolamine, might be a lipid similar to that investigated by Svennerholm & Thorin (1960).

Sphingomyelin in the brain of the rat reached its maximal concentration between 9 and 11 weeks of life and was paralleled by the total acid- and alkali-stable lipids (Table 3). The total amount per brain increased beyond this period because the rat brain increases in size for a period of a year or more (Donaldson, 1924). In the mouse the concentration of acid- and alkali-stable lipid is maximal at about 7 weeks after birth (Folch-Pi, 1955) and in the telencephalon of the rabbit, 17–18 weeks after birth; in this species the concentration subsequently falls (Edgar, 1957). The amount of unknown chloroform-soluble phospholipid appeared to reach a maximal concentration before the sphingomyelin, but the scatter of the values in Table 3 precludes a definite conclusion about this.

Experiments indicated that grey-matter sphingomyelin in the adult animal was capable of incorporating labelled phosphate, whereas that of the white matter was relatively inert as far as the phosphate moiety was concerned. That the adult-brain sphingomyelin as a whole is metabolically active is shown in Fig. 3, but it seems highly likely that this phosphate uptake is confined to sphingomyelin occurring outside the myelin sheath. The relatively inert nature of the sphingomyelin of the myelin sheath has been demonstrated by Davison *et al.* (1959), who showed that when [3-^{14}C]serine was injected into the developing rabbit it was incorporated into and retained by myelin sphingomyelin without significant loss for over 6 months. Cerebrosides and cholesterol, which are also characteristic myelin lipids, were shown to be likewise metabolically inert, in that loss of radioactive carbon was negligible once it had been incorporated during the initial accretion. It should be pointed out, however, that the whole acid- and alkali-stable phospholipid fraction was considered as sphingomyelin by Davison *et al.* (1959). The unknown components of this fraction have been shown in recent experiments (unpublished) to be quite significantly radioactive after the injection of labelled phosphate. Davison & Dobbing (1959) have in addition carried out experiments in which ^{32}P was injected into 16-day-old rats and the retention of the isotope by the brain phospholipid investigated. After 70 days a considerable proportion of the incorporated radioactivity remained in comparison with the negligible amount of radioactivity in the liver and kidney. These observations confirmed the early experiments of Changus, Chaikoff & Ruben (1938). In a subsequent investigation Davison & Dobbing (1960*a*) showed that there was a steady loss of radioactivity from the brain lipids until about 100 days after the injection, after which there was no measurable decline. From this they concluded that a proportion of the phosphate moieties of the brain phospholipid was metabolically inert, thus amplifying some conclusions drawn by Thompson & Ballou (1954, 1956). These authors showed that a considerable proportion of the phospholipids of the tissues of rats exposed to tritium when developing had long half-lives (220 days). They did not specifically study brain phospholipids and, as Davison & Dobbing (1960*a*) point out, the 'long-lived' components of Thompson & Ballou could be a mixture of inert and 'short-lived' components. These findings represent in some respects a revival of the concept of Folin (1905).

Discussing their experiments on the persistence of radioactivity in the myelin sheath as opposed to grey matter, Davison & Dobbing (1960*b*) conclude that phospholipids in this tissue are metabolically inert in adult life. It would certainly appear from our own experiments that sphingomyelin of the white matter of the adult rat brain is not particularly active as far as its phosphate metabolism is concerned, but it may not be completely inert. There are certainly great difficulties in interpreting the results obtained after the injection of labelled phosphate as far as brain tissue is concerned (Ansell & Spanner, 1960; Ansell, 1960) because of the chemical and morphological heterogeneity of the tissue. Even the relatively well-established inert brain component, cholesterol (Davison, Dobbing, Morgan & Payling-Wright, 1958) appears to be subject to turnover in adult life (McMillan, Douglas & Mortensen, 1957).

SUMMARY

1. Acid hydrolysis of cerebral phospholipids which are stable to mild acid and alkali hydrolysis has confirmed that sphingomyelin represents only a proportion of this fraction whereas the nature of the remainder is unknown.

2. Sphingomyelin has been determined in the brain tissue of rats and other species and the results compared with those obtained with other methods.

3. The concentration of true sphingomyelin in rat brain reaches its peak 9–11 weeks after birth.

4. The incorporation of labelled phosphate into rat-brain sphingomyelin reached a constant level

after 9 weeks. This metabolism was largely confined to sphingomyelin found in the grey matter.

Note added in proof. The predominant unknown phosphorus compound (X) has now been identified as glycerol-α-phosphate, which suggests that a glycerophospholipid survived the treatment preceding the methanolic hydrochloric acid hydrolysis [cf. Schmidt *et al.* (1959). *Amer. J. Dis. Child.* **97**, 691].

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The Action of Cellulolytic Enzymes from *Myrothecium verrucaria*

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Although an extensive literature is available describing cell-free cellulolytic preparations which attack soluble cellulose derivatives, there is a dearth of information on the enzymic breakdown of insoluble cellulose, and virtually nothing is known about the breakdown of undegraded cellulose of the type associated with native cotton fibres [For reviews see Siu (1951), Halliwell (1959)]. Despite the failure of cell-free preparations made from them to attack undegraded cellulose, truly cellulolytic organisms can quantitatively transform all types of cellulose, undegraded or otherwise, to soluble products.

The present report concerns an examination of the breakdown of an acid-swollen cellulose by culture filtrates from *Myrothecium verrucaria*. This substrate is an insoluble although degraded cellulose and was chosen firstly because the relatively mild method of preparation involves only small changes in the degree of polymerization (Walseth, 1952), and secondly only after having shown that our enzyme preparations produced significant solubilization of this substrate. Conditions governing the enzymic breakdown of insoluble cellulose are described below and compared with the enzymic hydrolysis of the soluble cellulose derivative, carboxymethylcellulose.

MATERIALS AND METHODS

Preparation of the culture filtrate

Cultures of *M. verrucaria* (I.M.I. 45541, Commonwealth Mycological Institute, Kew, Surrey), grown on the salt medium of Saunders, Siu & Genest (1948) with cellulose powder (Whatman) or de-waxed cotton fibres as substrate, were aerated at 28° by incubation in a reciprocal shaking machine operating at 60–90 strokes of 38 mm. horizontal movement per minute. After fermentation for 1–3 weeks most of the organisms and all of the residual cellulose were separated off by filtration through a sintered-glass filter (porosity 3). The filtrate was centrifuged at 54 000 *g* for 30 min. at 1° and subsequently passed through a sintered-glass bacteriological filter (porosity 5/3). In the later work the final filtration was found to be unnecessary by inoculation on cellulose–agar slopes. The cell-free culture supernatant or filtrate fraction was used as the source of cellulolytic enzymes. For enzyme assays the following average volumes were used: for method (*a*) 0·2 ml. of undiluted filtrate; for method (*b*) 0·1 ml. of filtrate diluted 50 times.

The solubilization of cellulose by the organism was determined by washing the mixture on the sinter with hydrochloric acid, aq. ammonia solution, Teepol and ethanol, followed by drying and weighing as described elsewhere (Halliwell, 1957*a*).

Substrates

Native cotton fibres. Texas cotton (kindly supplied by the British Cotton Industry Research Association) was de-waxed by treatment with ethanol, ether and finally with sodium hydroxide under nitrogen (Halliwell, 1957*a*).

Cellulose powder (*Whatman*). This was standard-grade ashless powder for chromatography.

Swollen cellulose. This insoluble preparation was made from Texas cotton fibres and also from cellulose powder (Whatman) by soaking in phosphoric acid for 2 hr. at 1° (Halliwell, 1957*a*).

Sodium carboxymethylcellulose. A soluble cellulose derivative, with a degree of substitution of about 0·5, was generously supplied as Cellofas B by Imperial Chemical Industries Ltd.

Other materials. These were: cellobiose and glucono-1→4-lactone (L. Light and Co. Ltd.); glucose (A.R.; Hopkin and Williams Ltd.).

Analytical procedure

Determination of the degree of hydrolysis of the soluble cellulose derivative carboxymethylcellulose. Hydrolysis of carboxymethylcellulose to 'reducing' sugars was followed by a modification of the colorimetric procedure of Park & Johnson (1949). The latter method was satisfactory with pure aqueous solutions of glucose, but not under assay conditions in the presence of phosphate buffer, owing to precipitation of reagents. This was overcome by substituting phosphoric acid for sulphuric acid in the ferric alum solution as follows: (*a*) 0·1 g. of ferric ammonium sulphate (A.R.) was dissolved in 1·5 ml. of conc. phosphoric acid (A.R., 90%) and 48·5 ml. of water; (*b*) to solution (*a*) was added a mixture containing 0·15 g. of Duponol (Duponol ME, dry, kindly given by E. I. du Pont de Nemours and Co. Inc., Wilmington, Del., U.S.A.) in 1·0 ml. of conc. phosphoric acid and 49 ml. of water.

Modified procedure for determination of 'reducing' sugar. The sample, containing 1–14 μg. of sugar, was neutralized (see below under enzyme assay) and made up to 3 ml. with water. Deproteinizing was unnecessary. After addition of 1 ml. of cyanide–carbonate solution (0·064% of potassium cyanide + 0·52% of sodium carbonate anhydrous) followed by 1 ml. of 0·05% potassium ferricyanide, the mixture was heated in a boiling-water bath for 15 min., transferred to a cold-water bath for 5 min. and treated with 2 ml. of ferric alum–Duponol–phosphoric acid reagent. Colours were

developed at room temperature for 15 min. before being read on a Spekker absorptiometer (Hilger) in a 1 cm. light-path with Ilford orange filters (no. 607, peak transmission 600 mμ).

Glucose and cellobiose gave straight-line calibration curves up to 14 μg. of sugar when analysed by the procedure above. Absorptiometer readings obtained after 15 min. of colour development increased slightly on further standing, but for periods of colour development up to 75 min. this error was eliminated by including a reagent control whose colour value increased by the same amount.

Determination of protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was used on a Spekker absorptiometer in a 1 cm. light-path with Ilford red filters (no. 608, peak transmission 700 mμ). As the copper sulphate–tartrate reagent tends to settle out on standing, tartrate was replaced by trisodium citrate dihydrate (A.R.). Crystalline bovine plasma albumin (Armour Laboratories) provided the standard reference protein. The procedure was used to follow protein formation in culture filtrates and to determine enzyme protein before and after dialysis.

Method of enzyme assay (a) with insoluble substrate, cellulose. As a result of preliminary experiments the following procedure was adopted. To about 26 mg. (or 1 mg. in earlier experiments) of cellulose [cotton fibres, cellulose powder (Whatman) or swollen forms of these celluloses] was added 1·3 ml. of acetate buffer, pH 5·5, (0·2N-acetic acid and 0·2N-sodium acetate), water and cell-free culture filtrate in a final volume of 4 ml., contained in 15-ml. R.B. heat-resistant, centrifuge tubes (110 mm. × 16 mm.; Measuring and Scientific Equipment Ltd.), or 25-ml. test tubes (125 mm. × 19 mm.; Griffin and George Ltd.). Test tubes were used when the residual cellulose was estimated after separation from the aqueous phase by means of a filter-stick. Centrifuge tubes were employed when cellulolysis was measured by increase in soluble carbohydrates formed from insoluble cellulose (see below). Cell-free culture filtrate–substrate mixtures were incubated for 1 hr. at 37°, and the enzyme was inactivated by addition of sufficient 0·6N-sulphuric acid (about 0·5 ml.) to lower the pH to 1·90–1·95. Enzyme and substrate controls were included.

Cellulose in the acidified suspensions was determined colorimetrically by two methods: (*a*) directly, the cellulose in its original tube being filtered, washed on the filter-stick and finally estimated with dichromate (Halliwell, 1958); (*b*) indirectly, from the amount of soluble carbohydrates present in the aqueous phase: the acidified assay medium containing cellulose in centrifuge tubes was centrifuged at 1300–1800 *g* for 3 min. at room temperature, and the supernatant passed through a sintered-glass crucible (porosity 3). The carbohydrates in the filtrate were quantitatively determined with dichromate, the values being calculated from a standard calibration curve prepared with glucose (Halliwell, 1960). The dichromate method is preferable to reducing-sugar methods for this purpose because it determines both 'reducing' and 'non-reducing' carbohydrates and thus gives a value much closer to the actual degree of solubilization (cf. Fig. 3).

The preliminary centrifuging was used to deposit the insoluble cellulose firmly, thus facilitating separation of most of the liquid phase. Filtration was essential to remove fine floating particles of insoluble cellulose, which failed to precipitate even on prolonged centrifuging.

Acidification of cellulose to pH 1·9 produced no solubilization of this substrate during the period required for centrifuging and filtration. Any solubilization that might occur under experimental conditions would be recognized in the substrate controls.

In some enzymic digests of cellulose the buffer solutions and volumes used were different from those given above for the standard assay with acetate. However, when 0·8–1·3 ml. of acetate buffer, pH 5·5, or 1·6 ml. of phosphate buffer (0·067M-disodium hydrogen phosphate + 0·067M-potassium dihydrogen phosphate, pH 5·5) or citrate buffer (0·1M-citric acid + 0·2M-disodium hydrogen phosphate, pH 5·5) were employed, enzymic activities on swollen cellulose powder were identical.

Filtrates obtained from enzymic incubations with cellulose in presence of phosphate or acetate buffers were examined by the indirect dichromate procedure (*b*) outlined above. As citrate buffer reduces dichromate, cellulolysis in such a medium was determined by direct procedure (*a*) above. Citrate was used only in measuring the pH optimum of the enzyme on swollen cellulose powder.

Method of enzyme assay (b) with soluble substrate, carboxymethylcellulose. To 0·3 ml. of 1% (w/v) carboxymethylcellulose was added 0·15 ml. of acetate buffer, diluted cell-free culture filtrate and water to 0·6 ml., final pH 5·5. The mixture was incubated at 37° for 1 hr. and the reaction terminated by addition of 0·25 ml. of 0·13% anhydrous sodium carbonate and 1 ml. of cyanide–carbonate (0·064% of potassium cyanide + 0·52% of sodium carbonate, see above). After addition of 2·15 ml. of water (total volume now 4 ml.) subsequent stages, commencing with the addition of 1 ml. of potassium ferricyanide, are identical with those described above (see 'Modified procedure for determination of reducing sugar'). The value, 0·25 ml. of 0·13% sodium carbonate, is experimentally determined as necessary to neutralize the enzyme reaction mixture to about pH 7·0, whereas the cyanide–carbonate adjusts the reaction of the neutralized mixture to about pH 10·7, thus attaining the required alkalinity of the standard glucose curve and simultaneously terminating enzymic activity. The diluted cell-free culture filtrate itself gave a negligible value when oxidized with ferricyanide, thus avoiding the necessity for deproteinization before sugar determination.

RESULTS

In the work described below the term cellulase is used to describe the activity in cell-free preparations attacking insoluble cellulose (swollen cellulose powder, swollen de-waxed fibres, cellulose powder and de-waxed fibres), with the proviso that a true cellulase (as yet not isolated) should produce extensive solubilization of undegraded cellulose (e.g. untreated fibres) in the manner of the parent micro-organism. Enzymic activity on the soluble cellulose derivative, carboxymethylcellulose, is referred to as carboxymethylcellulase.

Decomposition of de-waxed cotton fibres and of cellulose powder by cultures of Myrothecium verrucaria. Fig. 1 illustrates the capacity of the organism

to solubilize these less-degraded forms of cellulose. Continuous shaking was beneficial to extensive cellulolysis of both forms of cellulose and was also essential when profitable volumes of medium were required. In stationary cultures growth was much slower, and it was necessary to use smaller quantities of medium to avoid submerging the fungus.

Decomposition of de-waxed cotton fibres and of cellulose powder by enzyme filtrates of Myrothecium verrucaria. The degree of solubilization of these substrates by the cell-free culture filtrate (Table 1) is only a small fraction of that shown on the same substrates by the whole culture (Fig. 1). The initial rate of solubilization of undegraded fibres by the cell-free culture filtrate is comparable with that shown by whole organisms during growth, but the extent of solubilization by the enzyme preparation becomes constant after 22 hr., whereas with whole organisms, where growth is in progress, it increases to 80% in 16 days. It is first necessary to subject the cellulose to chemical treatment, such as swelling in acid, before cellulolysis by the culture filtrate becomes significant.

Activity of cellulase on swollen cellulose powder at different pH *values.* Citrate–phosphate buffer, covering the range pH 2–8 (Fig. 2) was used in mixtures, containing cell-free culture filtrate and

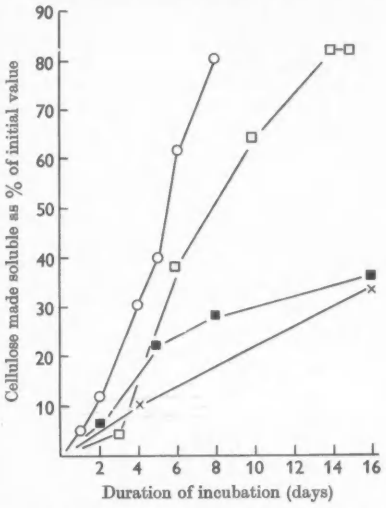

Fig. 1. Solubilization of cellulose by cultures of *Myrothecium verrucaria*. 50-ml. conical flasks containing (*a*) 50 mg. of cellulose powder (Whatman) in 10 ml. of salts medium, pH 6·6, or (*b*) 50 mg. of de-waxed Texas cotton fibres in 4·5 ml. of salts medium for shaken cultures (or in 1 ml. for stationary cultures) were inoculated with 1 ml. of a heavy spore suspension of the organism prepared from cultures of *M. verrucaria* grown on filter-paper strips on agar slopes in test tubes. The flasks were aerated by reciprocal shaking at 120 strokes of 50 mm. horizontal movement per minute for cellulose powder (or 60 strokes of 38 mm. for cotton fibres) and at 28° for the periods shown. Flasks were removed at intervals, the mycelium-cellulose residue was filtered on sintered glass, and washed and dried as described under Materials and Methods. Cellulose powder (Whatman) in shaken culture (○), in stationary culture (×); de-waxed cotton fibres in shaken culture (□), in stationary culture (■). Control flasks containing cellulose and medium (no inoculum) at 28° in shaken or stationary incubations showed no loss in weight of cellulose.

Table 1. *Solubilization of different forms of cellulose by cell-free filtrates from* Myrothecium verrucaria

Swollen cellulose powder (26 mg.) or swollen de-waxed Texas fibres (26 mg.) was incubated at 37° with 0·2 ml. of cell-free filtrate in acetate buffer, pH 5·5, for 1 hr. and 22 hr. Cellulose powder or de-waxed Texas fibres (5 mg.) was examined under the same conditions with 0·5 ml. of cell-free filtrate. Enzymic activity was estimated from the soluble carbohydrates formed. In the determination of carboxymethylcellulase activity by the standard method the cell-free filtrate was used at about one-twentieth of the final concentration of that with the swollen cellulose powder.

Type of cellulose	Degree of solubilization (% of initial wt.) in 1 hr.	22 hr.
Cellulose powder	—	3
De-waxed fibres	—	4
Swollen cellulose powder	11	56
Swollen de-waxed fibres	8	45
Carboxymethylcellulose (degree of hydrolysis)	0·4	—

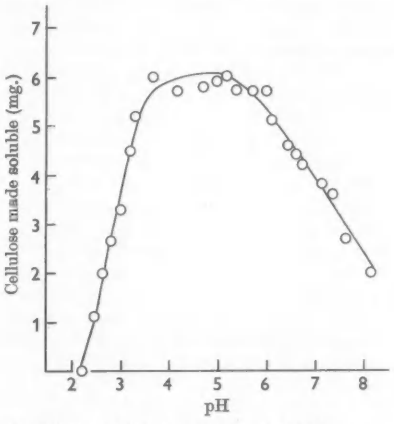

Fig. 2. Effect of pH on the activity of cellulase on swollen cellulose powder. Enzymic activity, measured by the standard method but in citric acid–phosphate buffer, was terminated by addition of sulphuric acid followed by filtration and determination of the residual cellulose with dichromate.

substrate, which were checked for constancy of pH during incubation. Optimum activity for the enzyme occurs about pH 5, with some hydrolysis still evident at pH 2·5. As enzymic activity at pH 5·5 is very similar to that at pH 5·0 the former pH was used in the standard assay medium in order to facilitate comparison with results of other workers in the field.

Effect of substrate concentration on enzymic activity. In the original cell-free culture filtrates the relationship between cellulose made soluble and initial weight of substrate indicated that saturation of the enzyme occurred at about 1 mg. of cellulose/4 ml. incubated. Activities were approximately proportional to the concentrations of enzyme used. In later experiments cell-free culture filtrates obtained from subcultures of the original organism provided different curves with saturation occurring at 27 mg. of cellulose/4 ml. of incubation mixture (Fig. 3).

The Figure also illustrates agreement between the two methods used to measure enzymic cellulolysis: (1) direct loss in weight of cellulose, and (2) production of soluble saccharides.

Carboxymethylcellulase activity in the culture filtrate was examined as in Table 2 with saturation being obtained at about 3 mg. of carboxymethylcellulose in the 0·6 ml. of assay volume.

The K_m values of the culture filtrate on swollen cellulose powder and on carboxymethylcellulose are calculated by the graphical method of Lineweaver & Burk (1934) (Fig. 4), and give values of

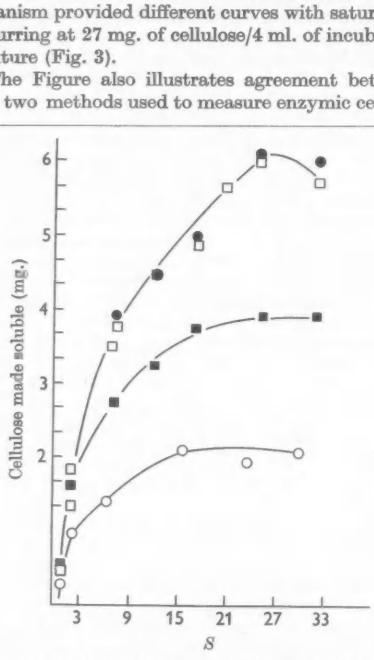

Fig. 3. Effect of weight of swollen cellulose powder on the amount solubilized by cellulase. Standard conditions of assay except that incubated mixtures, after inactivation with sulphuric acid, were filtered through sintered crucibles to obtain both the filtrate and residual cellulose. The latter was washed and determined gravimetrically. The filtrate was examined for the amount of soluble carbohydrates (see Methods section). Cellulose solubilized as determined from residual weight of cellulose, with culture filtrate A (0·5 ml.) (■), (1 ml.) (□); with another filtrate (0·2 ml.) (○). Cellulose solubilized as determined from soluble carbohydrates in the filtrate, with culture filtrate A (1 ml.) (●). *S*, Concn. of substrate (mg./4 ml.).

Table 2. *Effect of substrate concentration on the production of reducing sugar (as glucose) from carboxymethylcellulose*

Assay of the enzyme was made by the standard procedure. The cell-free culture filtrate was used at about one-fortieth of the concentration of that in Fig. 3 (■).

Carboxymethylcellulose in a total volume of 0·6 ml. (mg.)	Reducing sugar produced (as glucose) (μg.)
0·11	2·6
0·23	4·1
0·46	5·5
1·17	7·3
2·0	8·3
3·0	8·9
3·5	8·8
4·0	8·1

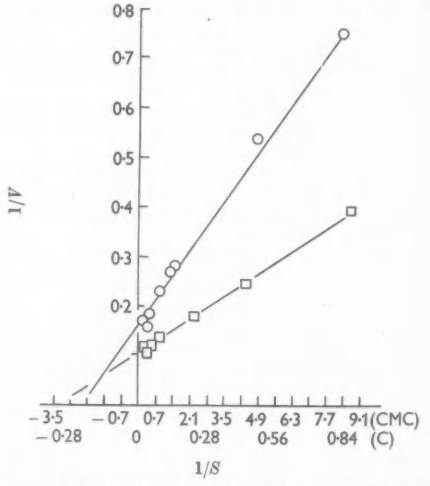

Fig. 4. Determination of the dissociation constant (K_m) of the enzyme–substrate complex for cellulase and carboxymethylcellulase by plotting the reciprocal of substrate concentration (S) against the reciprocal of rate of solubilization (V) of cellulose (for cellulase) or rate of formation of reducing sugar (for carboxymethylcellulase) from data of Fig. 3 (cellulase) and Table 2 (carboxymethylcellulase). ○, Cellulase (C); □, carboxymethylcellulase (CMC).

1 g. of cellulose/l. and 0·5 g. of carboxymethylcellulose/l. for the dissociation constant of the enzyme–substrate complex.

Effect of concentration of cell-free culture filtrate on the solubilization of swollen cellulose powder. At high enzyme concentrations the reaction velocity is not proportional to the amount of culture filtrate present (Fig. 5) probably owing in part to preferential enzymic attack on the more susceptible amorphous regions of the swollen cellulose. The effect was not due to the presence of a dialysable inhibitor as the dialysed culture filtrate possessed slightly less activity than the undialysed preparation when equal amounts of filtrate were used, based on protein content. Likewise, possible end products such as glucose or cellobiose produced no marked decrease in cellulolysis (see below). The effect could also indicate the presence of non-dialysable inhibitor in the cell-free culture filtrate.

Solubilization of cellulose as affected by the duration of enzymic activity. The rate of breakdown of swollen cellulose powder is almost linear for the first 45 min. but thereafter decreases progressively (Fig. 5). This presumably results from the early breakdown of the less refractory amorphous areas in cellulose and from the instability of the enzyme at 37° (see below) together with its increasing degree of unsaturation as the substrate is solubilized.

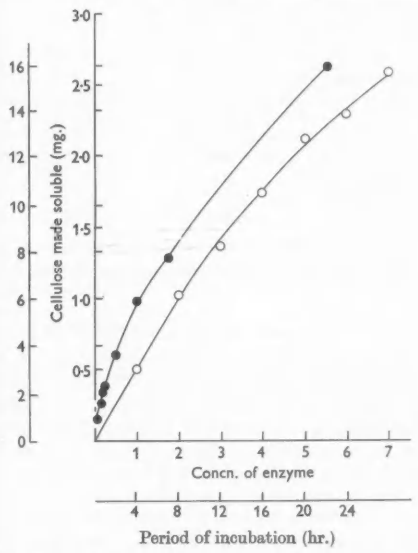

Fig. 5. Effect of enzyme concentration and of duration of incubation on the solubilization of swollen cellulose powder. Cellulase activity was measured and terminated by the standard method. Enzyme concentration was expressed as units in 4 ml., each unit corresponding to 0·05 ml. of cell-free culture filtrate. Soluble carbohydrates in the filtrate were determined with dichromate. Cellulose made soluble (0–3 mg.) as affected by concentration of enzyme (○). Cellulose made soluble (0–16 mg.) as influenced by period of incubation (●).

Effect of agitation on enzymic activity on cellulose and carboxymethylcellulose. Previous paragraphs have indicated that approximately 30-fold more cellulose was required to saturate later culture filtrates than was the case in earlier experiments. In an unsuccessful attempt to explain this difference, the action of cellulase (cell-free preparation) was examined with and without agitation on a Warburg apparatus operating at 107 complete strokes of 50 mm. horizontal movement per minute, when activity was found to be unaffected or increased only slightly by shaking. The same enzyme preparation examined under similar conditions with carboxymethylcellulose as substrate lost 72 % of its activity compared with an unshaken incubation (Table 3).

Activity of cellulase after heat treatment. Cellulase activity in the preliminary experiments (in which the culture filtrate was saturated with 1 mg. of cellulose) was completely terminated by immersing the reaction tubes containing filtrate and substrate in hot water (97°) for 20 min. In presence of the larger amounts of cellulose (27 mg.) required to saturate subsequent filtrates, the same heat treatment was less effective and allowed variable amounts of activity to continue. The effect was further examined by using shorter and longer periods of heating as follows: cell-free culture filtrates–cellulose mixtures incubated for 1 hr. were subjected to the following treatments: (*a*) immediate filtration (control), (*b*) immersion at 97° for 10 min. followed by incubation at room temperature (20°) for 30 min., (*c*) as in (*b*) but with a 30 min. heating period. In each case the cellulose was finally filtered, washed with hydrochloric acid, water, ethanol and dried at 105° before being weighed, when the degree of solubilization in (*b*) and (*c*) were 116 and 97 % respectively of that in (*a*).

It appears that one or more of the components of the enzymic preparation, possibly one concerned with the shorter but still insoluble chains, is not readily inactivated by brief periods of heat-treatment. Assays were more conveniently terminated by addition of dilute acid as described in the experimental section.

The filtrate was also examined for cellulase and carboxymethylcellulase activity by the standard procedures, after a preliminary incubation under assay conditions in the absence of substrate for 1 hr. and at different temperatures (Fig. 6). The results suggest that carboxymethylcellulase is less stable than cellulase, but in effect this is due to the experimental conditions of the respective assays

Table 3. *Effect of shaking on the activity of cellulase and carboxymethylcellulase*

Cellulase and carboxymethylcellulase activities were determined by the standard methods on swollen cellulose powder and on carboxymethylcellulose. Procedures for terminating and for estimating enzymic activity are described in the table. Tubes were shaken in a Warburg bath at 107 complete strokes of 50 mm. horizontal movement per minute and held in suitable positions (about 35° to the horizontal for cellulose) to obtain similar degrees of agitation with both enzymes.

Cell-free filtrate	Method of terminating enzymic activity	Method of estimating enzymic activity	Unshaken tubes: cellulose solubilized (% of initial weight)	Shaken tubes: cellulose solubilized (% of that solubilized in unshaken tubes)
		Cellulase activity		
1, 2	Immediate filtration and solvent washing (hydrochloric acid, water, ethanol and oven-drying)	Gravimetric, on residual cellulose	23	105
2	Addition of sulphuric acid to pH 1·95, rapid filtration under pressure and solvent washing (as above)	As above	21	98
3	Addition of sulphuric acid to pH 1·95, rapid filtration under pressure and solvent washing. Filtrate retained for next experiment	As above	15	106
3	Filtrate from previous experiment	Dichromate–sulphuric acid oxidation of soluble carbohydrates in the filtrate	15	108
4	Addition of sulphuric acid to pH 1·95, filtration under gravity, filtrate analysed	As above	15	98
		Carboxymethylcellulase activity		
			Unshaken tubes: reducing sugar formed (% of substrate)	Shaken tubes: reducing sugars formed (% of that in unshaken tubes)
3	Addition of carbonate–cyanide to pH 10·7	Ferricyanide determination of reducing sugars formed	0·4	28

rather than to inherent instability of that enzyme. Thus when the cell-free preparation itself was incubated in absence of substrate for 1 hr. (rather than under the respective conditions for the two enzymes) before removal of samples for the standard cellulase and carboxymethylcellulase assays, the latter enzyme appeared to be slightly more stable than cellulase (Fig. 6).

Effect of possible inhibitors on cellulase activity. Glucono-1 → 4-lactone or glucose at a final concentration of 2·5 mM had no effect on the rate of solubilization of cellulose by the cell-free culture filtrate, whereas the same molarity of cellobiose always produced slight inhibition, but never more than 10%. At the high concentration of 25 mM (18 mg. of glucose or 34 mg. of cellobiose in the presence of 26 mg. of cellulose) 15, 10 and 30% inhibition was obtained with gluconolactone, glucose and cellobiose respectively. The reaction medium containing carbohydrates equivalent to 1 mg. of glucose (dichromate method) obtained after incubation of cellulose and enzyme for 1 hr. was transferred to a fresh assay system and produced only 8% inhibition of cellulolysis, indicating that end products have negligible effect on cellulolysis.

Adsorption of cellulase by cellulose

Immediately after mixing cellulase and its substrate (swollen cellulose powder) the aqueous phase is relatively free from enzyme owing to the latter's adsorption, the extent of which depends on the initial quantity of cellulose present and on subsequent incubation of the mixture (Table 4).

In Table 4 separate samples were used for the 0 and 1 hr. experiments in order to avoid delays in filtration, but substantially similar results were achieved when the original sample of cell-free culture filtrate and cellulose (26 mg.) was successively centrifuged, the supernatant filtered off and the cellulose reincubated with fresh buffer solution. Thus after incubation for 0 and 1 hr., supernatant fractions containing 17 and 20% respectively of the original enzymic activity were obtained by successive centrifugings. The deposited cellulose was then washed once (or three

times) with dilute acetate buffer. On reincubation of the cellulose it was found to retain 49% of the enzyme originally added. All values are corrected for some adsorption of enzyme by the sinter itself and for the non-linearity of enzyme activity at high enzyme concentrations. It is noteworthy that 86% of the cellulase has survived 2 hr. incubation with substrate.

DISCUSSION

M. verrucaria produces almost complete solubilization of de-waxed cotton fibres (Fig. 1), whereas cell-free culture filtrates have an insignificant effect (4% solubilization) on this substrate. For cellulose powder prolonged incubation, with addition of further quantities of cell-free filtrate, provides a constant maximum of 26% solubilization achieved in 72 hr., comparable with the values given by our earliest preparations (Halliwell, 1957*b*). The latter, however, contrasted with present cell-free culture filtrates in being more active (requiring less enzymic material to produce this degree of solubilization) on cellulose powder, but less active on swollen cellulose powder. This activity was associated with a lemon-yellow colour present in the early culture media, but less evident in more recent metabolism fluids. Such apparent variance in chemical properties between cell-free preparations may also account for the different amounts of cellulose required to saturate the various cell-free culture filtrates described above.

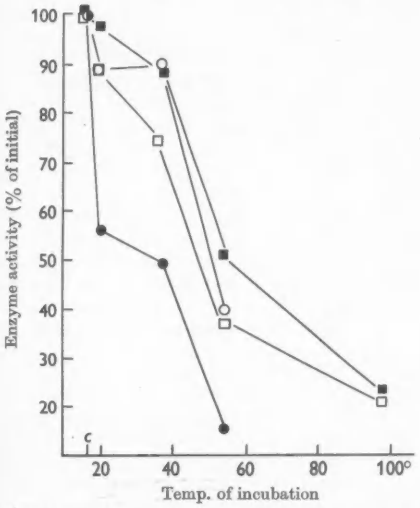

Fig. 6. Residual enzymic activity after incubation of the cell-free preparation in absence of substrates at different temperatures. Treatment *A*: cellulase (○), carboxymethylcellulase (●); the enzyme preparation was incubated in acetate buffer, pH 5·5, under the appropriate assay conditions for cellulase and carboxymethylcellulase (see Materials and Methods), but in the absence of substrate (cellulose or carboxymethylcellulose), at 20°, 37° or 54° for 1 hr. and then transferred to a bath at 37° followed immediately by addition of the respective substrates (cellulose or carboxymethylcellulose) and a further incubation of 1 hr. at 37°. Controls, assayed in the normal manner for 1 hr. at 37°, were taken to represent 100% enzymic activity (*c* on abscissa). Treatment *B*: cellulase (□), carboxymethylcellulase (■); the cell-free preparation was diluted fourfold in acetate buffer, pH 5·5, followed by (*a*) immediate assay (controls) on swollen cellulose powder or on carboxymethylcellulose, these activities being taken as 100% (*c* on abscissa), or (*b*) incubation at 20°, 37° or 54° for 1 hr. or 97° for 10 min. when samples were removed and assayed on swollen cellulose powder or, after dilution, on carboxymethylcellulose under the usual conditions (see Methods) for 1 hr. at 37°. Cellulase activities were determined from the amount of soluble carbohydrates formed.

After conversion of cotton fibres or cellulose powder into the swollen form, both the rate and extent of breakdown are increased (Table 1) so that almost complete solubilization (90%) can be achieved in 22 hr. by addition of larger amounts of enzyme.

The results show that the procedure described for the determination of activity on swollen, but insoluble, forms of cellulose powder and fibres is as rapid as it is on the soluble carboxymethylcellulose (Fig. 3, Table 2) under normal conditions of measurement. It thus seems preferable and possible to employ an insoluble cellulosic substrate in studies involving cellulase rather than to substitute carboxymethylcellulose.

Basu & Pal (1956) observed that carboxymethylcellulase preparations from several fungi, including *M. verrucaria*, were denatured (40%) if agitated during a 3 hr. assay procedure, whereas Myers & Northcote (1959) failed to obtain any inhibition of

Table 4. *Adsorption of cellulase by different amounts of cellulose*

ES (0) and ES (1) represent enzyme-swollen cellulose powder–buffer mixtures prepared in the standard manner (see Materials and Methods section) with mixing, followed by (*a*) immediate filtration (no centrifuging), ES (0), or (*b*) incubation for 1 hr. at 37°, ES (1), before filtration through a sintered-glass crucible. The enzyme in each filtrate was determined by assay on fresh cellulose–buffer as in Materials and Methods and the assay value (solubilization of cellulose) is expressed as a percentage of the enzymic activity of a control filtrate obtained in a similar manner to ES (0), but in absence of cellulose.

Cellulose (mg.)	Enzymic activity in filtrate (% of initial)	
	ES (0)	ES (1)
24	2	27
12	6	28
7	16	36

the same enzymic activity during a 20 min. shaking period with a preparation from snails. In our own work, a 1 hr. period of agitation produced severe loss (72%) of carboxymethylcellulase, but allowed cellulase activity to continue unimpaired (Table 3). The results achieved by Myers & Northcote (1959) are possibly due to the slower rate of shaking.

Fig. 6 indicates that carboxymethylcellulase loses activity more rapidly under its own particular assay conditions when devoid of substrate than does cellulase. On the other hand cellulase appears to be slightly less stable than carboxymethylcellulase when the cell-free culture filtrate, without additions, other than fourfold dilution in buffer, is incubated at different temperatures (in absence of substrate) before transfer to the two standard assay systems. Under their respective assay conditions but without added substrate the cell-free culture filtrate loses 50% of carboxymethylcellulase and 10% of cellulase activity during a 1 hr. incubation at 37°.

The possibility that end products resulting from enzymic activity on cellulose might inhibit further breakdown of this substrate does not exist under the present conditions. Only when glucose or cellobiose was added in very large amounts (18 and 34 mg. respectively in presence of 26 mg. of cellulose) did significant (10–30%) reduction in activity occur in 1 hr. End products of cellulolytic activity likewise failed to inhibit cellulase when transferred to a fresh enzymic assay on cellulose. Further, Fig. 5 also indicates that the accumulation of end products on prolonged incubation of enzyme with substrate for 22 hr. does not prevent solubilization from rising to at least 60% breakdown. Larger amounts of cell-free culture filtrate permitted 90% solubilization of cellulose in the same period. Gilligan & Reese (1954) have reported that relatively high concentrations of cellobiose were required to produce appreciable inhibition of the hydrolysis of hydrochloric acid- and phosphoric acid-treated, ground cotton by filtrates from *Trichoderma viride*.

Table 4 illustrates the high affinity of cellulose for its enzyme and probably accounts for the lack of any beneficial effect of agitation on cellulolysis. Cellulase is strongly adsorbed by its insoluble substrate and is slowly liberated during solubilization of the cellulose.

Cellulase (as examined on acid-swollen cellulose powder) is in fact a relatively stable enzyme, strongly adsorbed by its substrate and thus difficult to recover.

SUMMARY

1. The action of cell-free filtrates from *Myrothecium verrucaria* was examined on various forms of insoluble cellulose and on the soluble cellulose derivative, carboxymethylcellulose.

2. Unlike the parent organism which completely solubilizes undegraded forms of cellulose (de-waxed cotton fibres), cell-free culture medium has a negligible effect (4% solubilization) on the same substrate unless this is first swollen with phosphoric acid. After such treatment of cellulose, cellulolytic filtrates produce as much as 90% solubilization of swollen cellulose powder or of swollen de-waxed cotton fibres. Conditions governing the activity of the cell-free filtrates on swollen cellulose powder are described.

3. The preparation has an optimum activity on swollen cellulose powder at about pH 5.

4. Values of 1 g. of swollen cellulose powder/l. and of 0·5 g. of carboxymethylcellulose/l. were obtained for the Michaelis constants.

5. At pH 5·5 and temperatures between 20° and 54° the cell-free filtrate loses cellulase slightly more rapidly than carboxymethylcellulase activity. In contrast, under the appropriate assay conditions for the two activities, carboxymethylcellulase is denatured more rapidly than cellulase.

6. Agitation of cell-free filtrates in presence of cellulose has no marked effect on the degree of cellulolysis. With carboxymethylcellulose as substrate, however, agitation produces 70% inhibition.

7. Glucose, cellobiose or reaction products of enzymic cellulolysis fail to retard enzymic solubilization of cellulose unless present in amounts comparable with the initial weight of cellulose.

8. Cellulase in cell-free filtrates is strongly adsorbed on its insoluble substrate and is only slowly released as the cellulose itself is solubilized.

I sincerely thank Dr G. A. Levvy for his advice and encouragement and Mr G. Pratt for valuable technical assistance.

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Phosphoinositides

3. ENZYMIC HYDROLYSIS OF INOSITOL-CONTAINING PHOSPHOLIPIDS*

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Sloane-Stanley (1953) and Rodnight (1956) described the hydrolysis of ox-brain 'diphosphoinositide' preparations (Folch, 1949) by extracts of brain and other tissues. Dawson (1954) and Hokin & Hokin (1956) showed that the phosphoinositide fraction of several tissues had a high turnover rate, as measured by the incorporation of ^{32}P. The exact structure of the phosphoinositides involved is not known in every case, but it seems that the phosphate of phosphatidylinositol has a rapid turnover in several tissues (see Hawthorne, 1960*a*).

In view of these observations a study has been made of the hydrolysis of phosphatidylinositol and 'diphosphoinositide' by extracts of animal tissues. With the latter compound, Rodnight (1956) observed enzymic release of both organic and inorganic phosphate. This suggested that more than one enzyme was involved in the degradation and stimulated the present attempt to purify the enzyme catalysing the primary hydrolytic step in the breakdown of phosphoinositides.

Dawson (1959) has shown that phospholipase B preparations from *Penicillium notatum* and ox pancreas attack phosphatidylinositol. A preliminary report on this work has been published elsewhere (Kemp, Hübscher & Hawthorne, 1959).

MATERIALS AND METHODS

Analytical methods. The methods used for the determination of phosphate, nitrogen and glycerol have been described by Hawthorne & Hübscher (1959). Carboxylic ester was determined by the hydroxamate method of Stern & Shapiro (1953) and protein by the Gornal, Bardawill & David (1949) modification of the biuret method. Where solutions were very dilute, the ratio of absorption at 260 and 280 mμ was used to measure the protein content.

Paper chromatography. Methods used for the chromatography of glycerol, inositol, choline, ethanolamine and serine have been described before (Hübscher & Hawthorne, 1957). Inositol 1-phosphate and inositol 2-phosphate were separated by chromatography in propan-1-ol–aq. NH_3 soln. (sp.gr. 0·88)–water (6:3:1, by vol.) (Pizer & Ballou, 1959). Other solvents for the separation of inositol phosphate and glycerophosphate are described by Hawthorne & Hübscher (1959).

Ion-exchange columns. Details of the method for the separation of hydrolysis products of water-soluble phospholipid by ion-exchange chromatography have been published (Hawthorne & Hübscher, 1959).

Chromatography of glycerides. Total glycerides in extracts from enzymic digests were prepared by chromatography on alumina according to Trappe (1940). Mono- and diglyceride fractions were obtained from silicic acid columns as described by Barron & Hanahan (1958).

Ultracentrifugal analyses. Purified enzyme preparations were studied in the Spinco analytical ultracentrifuge.

Calcium phosphate gel. This was prepared as described by Keilin & Hartree (1938).

Preparation of phospholipid substrates

Phosphatidylinositol. This was prepared from ox or sheep liver by extraction according to Hawthorne (1960*b*), followed by chromatography on silicic acid by the method of Hanahan, Dittmer & Warashina (1957). The product was further purified on a second silicic acid column (Hanahan & Olley, 1958).

Diphosphoinositide fraction. This was prepared from ox brain by the method of Folch (1949). This fraction appears to contain more than one phosphoinositide.

Cardiolipin. This was obtained from ox heart by the method of Gray & Macfarlane (1958) and the sample was kindly supplied by Dr M. G. Macfarlane.

Phosphatidic acid. This was isolated from ox liver by the method of Hübscher & Clark (1960).

Phosphatidylcholine. This was prepared from the acetone-insoluble, alcohol-soluble fraction of ox-brain or ox-liver lipids and purified by chromatography on alumina according to Hanahan, Turner & Jayko (1951).

Phosphatidylethanolamine. This was isolated from egg yolk by the method of Rhodes & Lea (1957).

Phosphatidylserine. This was obtained from ox brain by the method of Folch (1948).

Preparation of phospholipid emulsions

The phospholipids were dissolved in chloroform and stored in the refrigerator until required. They were obtained in aqueous emulsion, essentially free from inorganic phosphate and other water-soluble contaminants, by the following method. A suitable volume of the chloroform solution was evaporated to dryness under reduced pressure and the residue dissolved in ether. The required volume of water was then added and the ether was evaporated under reduced pressure with constant shaking. When all the ether had been removed, the emulsion was transferred to a

* Part 2: Hawthorne, Kemp & Ellis (1960*a*).

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dialysis sac with an equal volume of 1% ethylenediaminetetra-acetic acid (EDTA) as its disodium salt. It was then dialysed for 72 hr. against distilled water, with frequent changes of water. It was difficult to remove all the inorganic phosphate from the diphosphoinositide fraction by this method. The purified emulsion was stored for use at 4°.

Assay of enzymic activity

The quantity of enzyme used was adjusted to give 20–40% hydrolysis of 12 μmoles of phosphatidylinositol added as an aqueous emulsion. Additions were made in the order: water, sodium acetate buffer (0·5 ml.; 0·4 M; pH 5·7), calcium chloride (40 μmoles), substrate and enzyme. The final volume was 4·0 ml. The tubes were kept for 20 min. at 0°, during which time they were occasionally shaken. Incubation was carried out for 1 hr. at 37°. For the blank experiment, substrate was omitted from the assay system. When the substrate was incubated in the absence of enzyme, neither water-soluble organic phosphate nor inorganic phosphate was liberated.

The reaction was stopped by placing the tubes in an ice bath, adding 0·8 ml. of 50% (w/v) trichloroacetic acid solution and mixing well. After 5 min., 2 ml. of an aqueous suspension of Norit A charcoal (12·5%, w/v) was added. This was required for the production of a water-clear filtrate. Cloudy filtrates could not be used for the determination of inorganic phosphate. The mixture was kept for a further 10 min. at 0°, filtered through a fluted Whatman no. 5 paper, and the filtrate analysed for inorganic and total phosphate.

Enzymic activity was expressed as follows: 1 unit of enzyme is the quantity required for the liberation of 1 μmole of water-soluble organic phosphate/hr. under the above-mentioned assay conditions, with phosphatidylinositol as substrate.

All preparations were carried out in a cold room at 4°. Samples to be assayed at once were kept in ice, otherwise they were quickly frozen and stored at −20°.

Preparation of subcellular fractions

Fresh rat liver was homogenized in ice-cold 0·25 M-sucrose (7 ml./g. of liver) for 45 sec. in a high-speed Waring mixer. The homogenate was then centrifuged for 10 min. at 2° and 730 *g*. The sediment was discarded and the supernatant centrifuged again at 35 000 *g* for 20 min. The material sedimented by this procedure was taken as the mitochondrial fraction. The supernatant was spun at 105 000 *g* for 45 min. to give the microsomal fraction. The resulting supernatant was retained.

All the precipitates were washed by resuspending in the original volume of 0·25 M-sucrose and centrifuging again at the same speed. They were then suspended in a small volume of 0·25 M-sucrose.

Acetone-dried powders

Acetone-dried powders of rat liver and intestinal mucosa and dog liver were prepared by the method of Kaplan & Lipmann (1948).

EXPERIMENTAL AND RESULTS

Evidence that the purified extract contained more than one enzyme acting on phosphatidylinositol was obtained only in the later stages of this work. In this section therefore the word enzyme is used in the singular to avoid confusion.

In preliminary experiments, homogenates of rat heart or liver were used. These were prepared by homogenizing the organs for 45 sec. in aqueous 0·9% sodium chloride solution (10 ml./g. of fresh tissue) in a high-speed Waring mixer. Acetate buffer was replaced in the assay system by the same molar concentration of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) hydrochloride buffer, pH 7·3. Calcium chloride solution was added to give a final concentration of 1 mM. With 1·0 ml. of heart or liver homogenate and with phosphatidylinositol as substrate, 0·5–1·0 μmole of total water-soluble phosphate was released/hr. Of this, between 10 and 20% was inorganic phosphate. Phosphatidylserine was hydrolysed at only about one-tenth of the rate of phosphatidylinositol.

It was considered likely that the inorganic phosphate was produced by the action of a phosphatase on a possible product of the phospholipase action, inositol monophosphate. To test this, the same assay system and source of enzyme was used, except that 7·0 μmoles of inositol 2-phosphate replaced the lipid substrate. A slow release of inorganic phosphate was observed (0·4–0·6 μmole/hr.). The first aim in purification of the phosphoinositide-splitting enzyme was the removal of this phosphatase.

Examination of subcellular fractions. Rat liver was chosen as a more convenient source of enzyme than rat heart owing to its larger size. Subcellular fractions were prepared as indicated above. Each fraction was dialysed against 0·9% sodium chloride solution for 24 hr. at 4° to remove sucrose and soluble phosphoric acid esters. Portions of these preparations equivalent in each case to 0·5 g. of fresh liver, and tris buffer, pH 7·3, were used in the assay. An increase in water-soluble organic phosphate was found with the supernatant fraction (1·3 μmoles/hr.) and the mitochondria (0·16 μmole/hr.). An increase of 0·1–0·2 μmole of inorganic phosphate/hr. was found in all fractions except the mitochondria, where the increase was 0·3 μmole/hr. In some experiments no release of organic phosphate was found with the mitochondrial fraction.

It was concluded that 60–70% of the activity was present in the supernatant fraction.

Distribution of enzymic activity. In an assay system with phosphatidylinositol as substrate and acetate buffer, pH 5·4, extracts of various tissues from different animals were tested for activity. Aqueous extracts of acetone-dried powders of dog liver, rat liver and rat intestinal mucosa all released water-soluble organic phosphate, but the specific activity of these extracts was lower than that observed with a fresh extract of rat liver. Activity was also detected in homogenates of sheep heart and rabbit liver as

well as in extracts of fresh brain, kidney and spleen of the rat. In all of these extracts, the specific activity and the total units per gram of wet tissue were lower than the corresponding values for extracts of fresh rat liver.

Purification of the enzyme. In the development of the method described below, several different techniques were tried, some of which proved unsuccessful. However, fractionation with ammonium sulphate gave good results. Furthermore, the enzyme could be absorbed by calcium phosphate gel and eluted again, the process giving a substantial purification. The following purification procedure was finally adopted.

Extraction of the enzyme. Four to eight livers were removed from male albino rats immediately after death and placed on ice. They were homogenized for 45 sec. in 10 mM-tris buffer, pH 7·3 (8 ml./g. of wet tissue), in the same high-speed mixer. The homogenate in this hypo-osmotic buffer was kept for 2 hr. at 4° to ensure extraction of the enzyme. The particulate matter was then removed by centrifuging at 31 000 *g* for 1 hr. To the supernatant, solid ammonium sulphate was added to give a final concentration of 25%. After 30 min. to 1 hr., the precipitate was separated by centrifuging at 22 000 *g* for 15 min.

First ammonium sulphate precipitation. The clear supernatant was made 40% saturated with respect to ammonium sulphate and kept for about 1 hr. at 0°. The precipitate was collected by centrifuging at 2700 *g* for 10 min. and dissolved in 5 mM-tris buffer, pH 7·3, to give a solution containing 5 mg. of protein/ml. If necessary it could be stored overnight at −16° before the next stage was reached. With a glass-electrode pH meter, the solution was adjusted to pH 6·8 by adding a few drops of 0·1 N-acetic acid.

Calcium phosphate-gel treatment. Concentrated calcium phosphate gel (23 mg. of tricalcium phosphate/ml. of water) was added (1 mg. of dry gel/mg. of protein) and the mixture stirred gently for 10 min. in an ice bath. It was then centrifuged for 5 min. at 750 *g*. The supernatant was decanted and the gel resuspended in 1·8 mM-sodium acetate–0·18M-ammonium sulphate solution, pH 7·0. One-half volume of sodium acetate–ammonium sulphate solution was used for elution per volume of solution from which the enzyme was absorbed. The gel was stirred with the solution as before for 10 min. After centrifuging at 750 *g* for 5 min. and removal of the supernatant, the gel was eluted in the same way with 0·5M-sodium acetate–ammonium sulphate solution, pH 7·0. The gel was again separated from the supernatant by low-speed centrifuging.

Second ammonium sulphate precipitation. To the eluate obtained with 0·5M-sodium acetate–ammonium sulphate solution, 16 g. of solid ammonium sulphate was added/100 ml. After 30 min. the precipitate was collected by centrifuging at 2700 *g* for 10 min. and dissolved in 10 mM-tris buffer, pH 7·3. The solution was stored at −16°.

A flow sheet of the purification procedure is given in Table 1. It will be seen that 49-fold purification of the enzyme was achieved with a recovery of 29%. The original supernatant contained all the activity detected in the homogenate. When stored at −16° the final enzyme preparation was stable for about 10 days.

Properties of the purified enzyme preparation. The purified preparation of the enzyme was readily soluble in the assay system buffer to give a completely clear, almost colourless solution. An examination of the preparation in an analytical ultracentrifuge, in 60 mM-phosphate buffer, pH 6·9, containing 150 mM-sodium chloride, revealed four components with molecular weights of (*a*) 60 000 (39·2%), (*b*) 200 000 (39·2%), (*c*) 400 000 (6·2%) and (*d*) 500 000 (15·4%). No attempt was made to correlate the enzymic activity with any one of the components. Assuming that each of the four components was a pure protein, it can be concluded from these data that the preparation was at least 6% and at most 39% pure.

The substrate was added to the assay system as an aqueous dispersion rather than a true solution and thus the enzyme–substrate complex was probably one in which a lipid micelle was involved rather than a single molecule of phosphatidylinositol. The effect of increasing the concentration of phosphatidylinositol is demonstrated in Fig. 1.

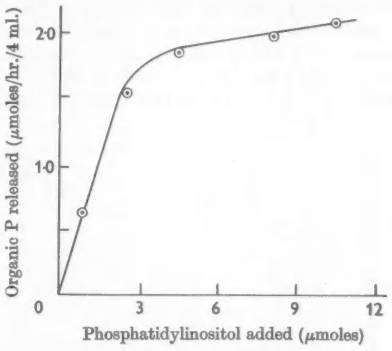

Fig. 1. Effect of substrate concentration on rate of enzymic hydrolysis. Assay system was as described in the text. Enzyme: second ammonium sulphate precipitate, 1 mg. of protein/4 ml.

Table 1. *Flow sheet for enzyme purification*

Step	Total vol. (ml.)	Protein (mg.)	Units/mg. of protein	Total units	Purification	Recovery (%)
Supernatant	225	2860	0·148	422	3·3	100
First ammonium sulphate ppt.	125	625	0·490	307	10·9	73
After calcium phosphate-gel treatment*	73	97	0·830	81	18·5	19
Second ammonium sulphate ppt.	73	55	2·180	120	48·5	29

* The activity of this preparation was difficult to determine accurately, possibly because of the presence of excess of Ca^{2+} ions.

There was relatively little increase in reaction rate at substrate concentrations above 3 mM.

When the data of Fig. 1 were treated according to Lineweaver & Burk (1934) a linear relationship was obtained which indicated a K_m value of $3{\cdot}3 \times 10^{-4}$ at pH 5·6. A determination of the Michaelis–Menten constant with a less pure enzyme preparation (first ammonium sulphate precipitate) gave K_m $4{\cdot}4 \times 10^{-4}$, the optimum substrate concentration being $3{\cdot}7 \times 10^{-3}$ M. Further evidence that the assay of the enzyme was carried out under zero-order conditions was obtained from measurements of the rate of release of water-soluble organic phosphate under the conditions of assay. Thus in the presence of 8 mg. of protein from the first ammonium sulphate precipitate organic phosphate was released without initial lag and at a constant rate of 0·9 ($\pm$0·1) μmole/10 min. for a period of 50 min.

At low concentrations of protein, the release of water-soluble organic phosphate was proportional to the amount of protein added. Under the assay conditions the rate of release of water-soluble organic phosphate from 8 μmoles of phosphatidylinositol was 1·85 ($\pm$0·25) μmoles/hr./mg. of protein of the second ammonium sulphate precipitate when the protein concentration was varied from 0·5 mg. to 2·0 mg./4 ml. of incubation mixture.

The change in activity with the pH of the reaction mixture is shown in Fig. 2. The optimum pH value was 5·7 $\pm$ 0·2. A second smaller optimum was present at pH 6·9 $\pm$ 0·2. The smaller optimum was consistently observed in varying stages of purification of the enzyme, though the relative activity per milligram of protein of the two optima varied from preparation to preparation under otherwise identical assay conditions. This might suggest the presence of two enzymes acting on the same substrate.

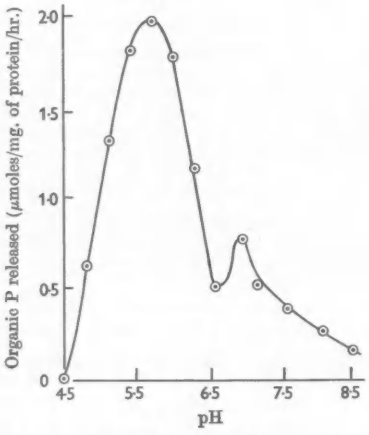

Fig. 2. Effect of pH on rate of enzymic reaction. Assay system was as described in the text, with phosphatidylinositol as substrate and the second ammonium sulphate precipitate.

The requirement for metal ions depended largely on the pretreatment of the substrate and enzyme. Thus when an aqueous undialysed emulsion of the substrate and an undialysed enzyme were used, no increase in the rate of reaction was brought about by the addition of Ca^{2+} ions, but an inhibition was obtained at higher concentrations of calcium chloride. These effects are shown with a less purified enzyme in Fig. 3 (*a*). Although the phospholipid used had been prepared by chromatography on two consecutive columns of silicic acid, substantial amounts of Ca^{2+} ions must have been carried through the purification procedure. The requirement for Ca^{2+} ions could readily be shown by using as substrate a preparation which had previously been dialysed for long periods of time against a dilute buffer containing EDTA. The enzyme preparation also contained a small but significant amount of Ca^{2+} ions. Since the enzyme was not stable during dialysis, a complete recovery of activity by the addition of Ca^{2+} ions was difficult to demonstrate. A typical activation of the enzymic reaction by Ca^{2+} ions with a dialysed substrate, but undialysed enzyme (the second ammonium sulphate precipitate), is indicated in Fig. 3 (*b*). Under these conditions, optimum concentrations of calcium chloride were between 5 and 16 mM. Since the concentration of phosphatidylinositol was 3 mM, the molar ratio of Ca^{2+} ions to substrate at maximal activity lies therefore between 1:0·6 and 1:0·2. Of a number of other bivalent ions tested, only barium and magnesium

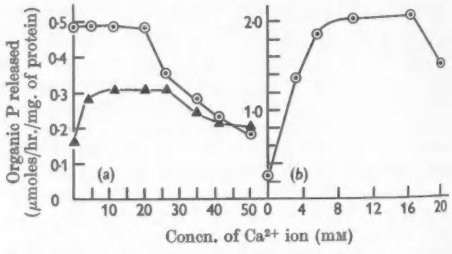

Fig. 3. Activation of enzyme by calcium. (*a*) First ammonium sulphate precipitate; usual assay system. ⊙, Undialysed enzyme; ▲, EDTA-dialysed enzyme. Lipid substrate not dialysed. (*b*) Undialysed second ammonium sulphate precipitate. Assay system was as described in the text but with only 8 μmoles of phosphatidylinositol (dialysed after addition of excess of EDTA).

showed a similar effect, yet in a lesser degree (see Table 2). Some other compounds were tested in the presence of optimum amounts of Ca^{2+} ions to obtain more information about possible active sites of the enzyme. There was a complete inhibition by low concentrations of *p*-mercuribenzoic acid. Neither glutathione nor cysteine when added in the absence of this inhibitor brought about an increase in the rate of reaction. Fluoride ions at 10 mM concentration gave a small, but significant, stimulation in the presence of 10 mM-Ca^{2+} ions. Owing to the insolubility of calcium fluoride addition of fluoride might be expected to decrease the effective Ca^{2+} ion concentration to 5 mM. This decrease would not itself cause the observed increase in rate [see Fig. 3 (*b*)].

The substrate specificity of the enzyme with a less pure preparation was reported previously (Kemp *et al.* 1959). These experiments were repeated with the second ammonium sulphate precipitate prepared as described in the present work. Very similar results were obtained. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, cardiolipin and phosphatidic acid were not attacked by the enzyme. On a molar basis, 'diphosphoinositide' gave twice as much water-soluble organic phosphate as did phosphatidylinositol.

The primary enzymic cleavage of phosphatidylinositol with release of water-soluble phosphate esters could lead to the formation of either glycerylphosphorylinositol or inositol monophosphate. The simultaneous determination of water-soluble organic phosphate liberated and of acyl values in the reaction mixture should therefore give some indication of the hydrolytic pathway.

The results of a study of the release of organic phosphate and fatty acid are given in Fig. 4. After incubation for 60 min. 6·2 μmoles of phosphoric acid ester, but only 3·2 μmoles of fatty acid, were liberated. Since the formation of 1 mole of glycerylphosphorylinositol is accompanied by the liberation of 2 moles of fatty acid, the release of 3·2 μmoles of fatty acid would correspond to the formation of 1·6 μmoles of glycerylphosphorylinositol. If the preparation contained a lipase hydrolysing the diglyceride formed upon release of inositol monophosphate from phosphatidylinositol, the above calculation would not hold. Although small amounts of monoglyceride were found among the reaction products, the separation of water-soluble phosphates described below suggests that the decrease in acyl values is mainly due to the formation of glycerylphosphorylinositol.

Identification of the reaction products. A hydrolysis was carried out with phosphatidylinositol as substrate and about 50 times as much enzyme as was usually taken for an assay. The other components of the assay system were increased in amount proportionately. The reaction was stopped after 60 min. by addition of trichloroacetic acid to give a final concentration of 5%. The precipitate was collected by centrifuging and extracted with ether. The aqueous supernatant was extracted with ether to remove trichloroacetic acid. A stream of air was passed through the aqueous solution to remove dissolved ether. After passing the solution through Zeo-Karb 225 (H^+ form), the volume was adjusted to 1 l., sodium tetraborate being added to give a final concentration of 5 mM. This solution of

Table 2. *Effect of bivalent metal ions at* pH 5·5

	Relative rate		
Concn. (mM) ...	2	6	10
Metal added			
None	100*	100*	100*
Ca^{2+}	325	500	500
Mg^{2+}	—	150	150
Ba^{2+}	50	100	185
Ni^{2+}	50	40	30
Zn^{2+}	25	—	15
Co^{2+}	70	55	60
Fe^{2+}	55	18	18
Pb^{2+}	26	37	20
Cu^{2+}	25	—	—

* Arbitrarily set at 100.

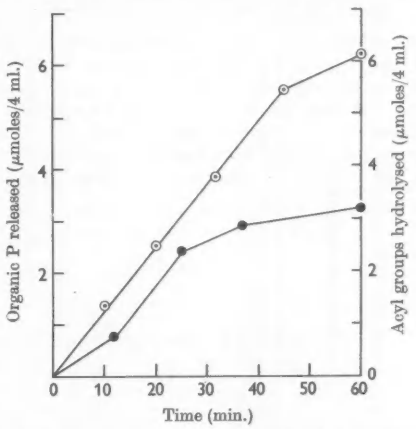

Fig. 4. Time study of water-soluble phosphate and fatty acid release. ⊙, Release of phosphate; ●, hydrolysis of acyl groups. Assay system was as described in the text with omission of charcoal. The trichloroacetic acid precipitate was spun down, washed once with water and extracted with excess of chloroform–methanol (1:1, v/v). The extract was dried and taken up in ethanol–ether (3:1, v/v) for acyl ester determinations. For the calculation of decrease in ester value, the zero-time result was taken as 100%.

phosphate esters was applied to a Nalcite SAR resin column (see Materials and Methods section) and, after the column was washed with 250 ml. of 5 mM-borate, elution was carried out as outlined in Fig. 5. Two main peaks were obtained (1 and 3), which corresponded in their position to glyceryl-phosphorylinositol (peak 1, 41 % of total phosphate) and inositol monophosphate (peak 3, 47·5 %). The first peak contained glycerol, phosphate and inositol in molar ratios 1·0:1·0:0·85. The identity of inositol monophosphate in peak 3 was confirmed by paper chromatography in propan-1-ol–ammonia (sp.gr. 0·88)–water (6:3:1, by vol.). By this method it was shown that the ester isolated was myoinositol 1-phosphate contaminated with about 5 % of the 2-isomer.

The ether extract of the precipitate was washed well with water to remove trichloroacetic acid and twice with 5 % sodium bicarbonate solution to extract free fatty acids. It was then washed with water once more, dried over anhydrous sodium sulphate and chromatographed on silicic acid by the method of Barron & Hanahan (1958). The mono- and di-glyceride fractions weighed 31 and 27 mg. respectively. Assuming a molecular weight of 285 for the fatty acids, analysis for glycerol and acyl groups gave the expected value for acyl groups, but the values obtained for glycerol content were somewhat low, possibly due to incomplete hydrolysis. The diglyceride fraction had a molar ratio of fatty acid to glycerol of 2·5 whereas the corresponding value for the monoglyceride fraction was 1·3. The sodium bicarbonate washings were acidified and the fatty acids were extracted with ether. The ether was removed *in vacuo* and the residue was dried over phosphorus pentoxide. It was then left over solid sodium hydroxide overnight in a vacuum desiccator to remove any volatile acids. The weight of the residue was 50 mg. No phosphate was present in this fraction.

DISCUSSION

The method used in the present investigation for assaying the enzymic release of water-soluble phosphoric acid esters from phosphatidylinositol has an obvious disadvantage. It determines the total esters released rather than a particular ester. Consequently, if various fractions are assayed during the course of an isolation procedure, little information is obtained about the relative concentration of the two or more enzymes which may be hydrolysing phosphatidylinositol. For further studies on the isolation of enzymes hydrolysing phosphatidylinositol, more specific assay systems will need to be devised.

Our purified preparation had, in fact, two independent hydrolytic actions. This was borne out by

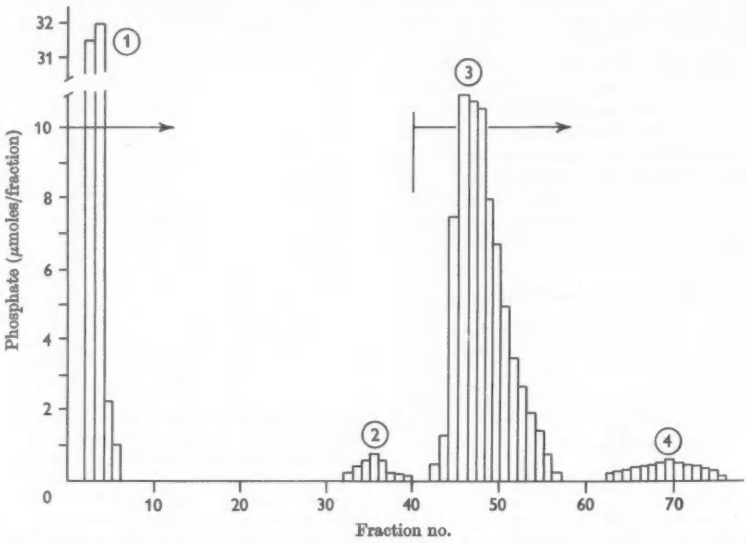

Fig. 5. Chromatography of hydrolysis products from enzymic reaction. Nalcite SAR resin. Eluting mixtures: fractions 1–39, sodium tetraborate (5 mM)–ammonium formate (60 mM); fractions 40–80, sodium tetraborate (5 mM)–ammonium formate (150 mM). Probable identities of peaks are given in the text.

various determinations: the simultaneous release of roughly 6 moles of phosphoric acid ester and 3 moles of fatty acid excluded the formation of inositol monophosphate via glycerylphosphorylinositol. The isolation of glycerylphosphorylinositol, inositol monophosphate, free fatty acid and lower glycerides from the reaction mixture would support this view. Since monoglyceride was found, a lipase may also be present which hydrolyses the diglyceride initially released by the phospholipase. The presence of two pH optima is also of interest in this connexion. The ratio of the activities at the two pH optima varied from preparation to preparation, with identical conditions for assaying. This might be explained by the presence of two enzymes hydrolysing phosphatidylinositol.

The two pathways for the hydrolysis of phosphatidylinositol are given in Fig. 6. It is generally assumed (e.g. Dawson, 1957) that the breakdown of glycerophosphatides in animal tissues occurs as outlined in pathway (A). In fact, the breakdown of a phospholipid in animal tissues by pathway (B) seems to have been described only by Dawson (1959), who showed that a lecithinase B preparation from ox pancreas released inositol monophosphate from phosphatidylinositol. This preparation hydrolysed lysolecithin more rapidly than phosphatidylinositol, whereas the enzymes described in the present work appear specific for phosphoinositides. The pancreas and liver enzymes have similar pH optima and requirement for Ca^{2+} ions. The liberation of phosphorylcholine from lecithin has been reported (Macfarlane & Knight, 1941; Long & McGuire, 1953; Chu, 1949; Hanahan & Vercamer, 1954). However, the sources of the enzymes concerned were extracts of *Bacillus cereus*, *Bacillus mycordes* and *Clostridium perfringens*.

The primary products of the hydrolysis of phosphatidylinositol by pathway (B) are a diglyceride and inositol monophosphate. The diglyceride could either be further degraded to glycerol and free fatty acids by the action of a lipase or it might be used for biosynthesis of phospholipids. D-$\alpha\beta$-Diglyceride is known to be a precursor of phosphatidylcholine and phosphatidylethanolamine (Kennedy, 1956). Free inositol monophosphate has been found in liver (Hübscher & Hawthorne, 1957). The enzyme described here probably accounts for its presence, especially since the biosynthetic pathway to phosphatidylinositol does not seem to include inositol monophosphate as an intermediate (Agranoff, Bradley & Brady, 1958; Paulus & Kennedy, 1960).

In the present investigation it was shown that inositol 2-phosphate is hydrolysed by crude extracts of liver tissue. Liver phosphatidylinositol has the myoinositol 1-phosphate structure (Pizer & Ballou, 1959; Hawthorne, Kemp & Ellis, 1960*b*). The liberation of inorganic phosphate from phosphatidylinositol by crude extracts or subcellular particles of rat liver suggests that the 1-isomer is also hydrolysed. It is not known whether the enzyme catalysing this reaction is one of the unspecific phosphomonoesterases or a specific phosphatase. Pileggi (1959) described a phytase from animal tissues which hydrolysed phytic acid completely, giving free inositol.

In pathway (A) the action of phospholipases A and B gives glycerylphosphorylinositol. The enzyme preparation did not attack lecithin or any of the other glycerophosphatides, though the less pure preparation (Kemp *et al.* 1959) hydrolysed lysolecithin at about 10% of the rate for phosphatidylinositol. It seems, then, that our enzymes are different from the phospholipases in a particulate preparation of the intestinal mucosa of the rat

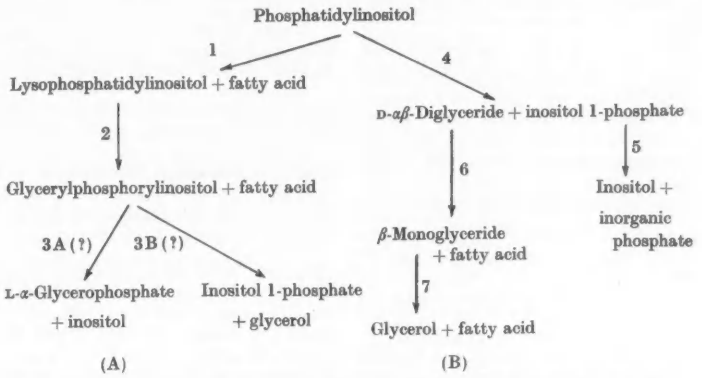

Fig. 6. Two possible pathways for the breakdown of phosphatidylinositol in liver.

(Epstein & Shapiro, 1959). These authors describe lecithinases A and B, which did not require Ca^{2+} ions for their activity. A phospholipase prepared from *Penicillium notatum* also attacked phosphatidylinositol, giving glycerylphosphorylinositol (Dawson, 1959). This enzyme was not activated by calcium, had optimum pH 3·3 and hydrolysed phosphatidylinositol more slowly than lysolecithin. The latter compound inhibited the breakdown of phosphatidylinositol, whereas the liver enzyme was unaffected (Kemp *et al.* 1959). In the presence of phosphatidylinositol or certain other anionic amphipathic molecules, the *P. notatum* enzyme would also attack lecithin (Bangham & Dawson, 1960). Glycerylphosphorylinositol might be further broken down by a diesterase to give either glycerophosphate or inositol monophosphate or both. The diesterases from liver (Dawson, 1956) or nervous tissue (Webster, Marples & Thompson, 1957) gave glycerophosphate and choline as products of the hydrolysis of glycerylphosphorylcholine.

The naming of enzymes attacking phosphoinositides is not easy. The enzyme removing diglyceride from phosphatidylcholine is referred to as lecithinase C by many workers, after the original suggestion of Macfarlane & Knight (1941). The same enzyme is also called lecithinase D (Hanahan, 1957). Since the enzymes described in the present study appear to be specific for phosphoinositides, the name phosphoinositidase seems appropriate for both. The enzyme removing diglyceride would then be a phosphoinositidase D.

SUMMARY

1. A soluble enzyme which hydrolyses phosphoinositides has been purified 50-fold from rat liver.

2. It hydrolyses phosphatidylinositol and the diphosphoinositide fraction from brain, but does not attack phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidic acid or cardiolipin.

3. For optimum activity Ca^{2+} ions are required. Two pH optima and other properties of the preparation are described.

4. Two independent hydrolytic actions were found to be present in the preparation leading to the formation from phosphatidylinositol of inositol monophosphate (phosphoinositidase C) and of glycerylphosphorylinositol (phosphoinositidases A and B).

We would like to thank Profesosr A. C. Frazer for his support and interest in this work. Our thanks are also due to the Department of Experimental Pathology, University of Birmingham, for analysis of enzyme preparations in the ultracentrifuge. Mrs B. Evans and Miss M. Bason provided skilled technical assistance. This work was supported by grants from the University of Birmingham and from the Department of Scientific and Industrial Research to one of us (P.K.).

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Biosynthesis of Ubiquinone in the Rat

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Rüegg *et al.* (1959) and Linn *et al.* (1959) have reported that rat liver contains two ubiquinones with C_{45} and C_{50} side chains (coenzymes Q_9 and Q_{10} respectively), the former predominating. Experiments in this Laboratory confirmed the presence of both these ubiquinones in our stock-colony rats and also showed the presence of traces of ubiquinones with side chains containing 40 and 35 carbon atoms (Lawson, Mercer, Glover & Morton, 1960). Similar observations have also been made independently by others (Diplock, Edwin, Green, Bunyan & Marcinkiewicz, 1960).

The occurrence of at least four ubiquinones in the rat raises the question of their origin. One or more may come from the diet, some components of which are known to contain ubiquinone (Page, Gale, Koniuszy & Folkers, 1959) and others may be formed by intestinal micro-organisms. The similarity between the ubiquinones and vitamins K_2 suggests that the rat possibly makes use of a preformed substituted benzoquinone moiety, to which it may attach one of a series of polyisoprenoid units in a manner similar to that claimed by Martius & Esser (1958) for the utilization of 2-methyl-1:4-naphthoquinone in the synthesis of vitamin K_2 (with a polyisoprenoid side chain). It has already been shown that [2-^{14}C]mevalonate (Gloor & Wiss, 1959*a*) and [1-^{14}C]acetate (Dialameh & Olson, 1959) can be incorporated *in vivo* into ubiquinone by the rat. The radioactivity was only in the side chain (Wiss, 1959; Gloor, Schindler & Wiss, 1960) so the aromatic nucleus is presumably independently available.

The origin of this substituted quinone portion of the molecule in the animal body is still unknown, although in micro-organisms similar quinones such as aurantiogliocladin (Birch, Fryer & Smith, 1958) and fumigatin (D. E. M. Lawson, N. M. Packter and J. Glover, unpublished observations) have been shown to be derived from [^{14}C]acetate. A number of aromatic compounds have been tested for precursor function. Gloor & Wiss (1959*a*) showed that [^{3}H]methylfumigatin (3:4-dimethoxy-2:5-toluquinone) was not incorporated into ubiquinone in the rat. Again, the nucleus of vitamin E apparently cannot be utilized (Gloor & Wiss, 1959*a*; Alaupovic & Johnson, 1959). Further, vitamin E deficiency does not appear to affect the concentration of ubiquinone in rat tissues (Morton & Phillips, 1959), although more recently results have been obtained which are at variance with this and suggest that ubiquinone levels are lowered (Green, Edwin, Diplock & Bunyan, 1960).

The aim of the study was to determine the portions of the ubiquinone molecule which can be synthesized by the various tissues of the rat. Secondly, it would be useful to know if a particular quinone administered orally can be stored unchanged and utilized in the animal or converted into the other homologues. In the present work, [2-^{14}C]acetate was administered intraperitoneally or orally to rats and the specific activities of ubiquinone from intestine, skin and liver were determined. Several experiments have been carried out to measure the incorporation of [2-^{14}C]mevalonate into ubiquinone formed *in vitro* during incubation for 3–9 hr. of everted intestinal sacs (Wilson & Wiseman, 1954) of the rat. The absorption of ubiquinone-45 administered orally in single doses has been studied in the rat and the effect on endogenous ubiquinone-45 levels of adding ubiquinone-50 to the diet has been explored for rat liver and intestine.

EXPERIMENTAL

Animals. Hooded rats (average weight 160 g.) from the stock colony were used in all experiments. Females were used in Expts. I, II, III, VIII and IX, and males were used in the remainder. The females normally contain a slightly higher level of ubiquinone in the liver than the males (Hemming, 1958; Green *et al.* 1960). The animals were maintained on food cubes (No. 4 diet, British Extracting Co., Port Sunlight). Special diets, as free as possible from fat-soluble pigments, were, however, substituted when test substances were being administered. This ensured that the spectroscopic examination of intestinal lipids was not made unnecessarily difficult.

Diet no. 1 contained (%): sucrose, 65; casein, 18; Yestamin, 8 (English Grains Co. Ltd., Burton-on-Trent); arachis oil, 5; salt mixture 4 (Osborne & Mendel, 1913); choline chloride, 0·1.

Diet no. 2 contained (%): sucrose, 70; casein, 20; arachis oil, 5·4; salt mixture 4·3; synthetic vitamin B mixture, 0·3 (Horner, 1958). Vitamin A was given in the form of a premix, each rat receiving 80 i.u. per week. This diet was low in ubiquinone-45 and was used for feeding experiments with ubiquinone-50. Ubiquinone could not be detected in the arachis oil, 20 g. of which were saponified and the unsaponified part was chromatographed on alumina as described below.

Dosing. In Expt. I, three doses of [2-^{14}C]acetate (10·4 μc in 0·5 ml. of 0·9% NaCl) were injected intraperitoneally on successive days to each of three rats from the stock colony. In Expt. II, two doses of [2-^{14}C]acetate (16·4 μc each) dissolved in water, were taken up on a little dry cellulose powder and admixed with about 3 g. of diet no. 1. The mixture was then formed into small pellets which the starving animals ate readily without loss.

The remainder of the daily supply of diet (about 10 g.) was then given to the animals. In Expt. VIII, the absorption and storage of single doses of ubiquinone were examined with nine female rats from the same litter. Each of four in the test group were given 4·7 mg. of ubiquinone-45 dissolved in a little arachis oil. After 24 hr. the test and five control animals were killed, and their tissues examined for ubiquinone.

During this work the stock diet contained mainly ubiquinone-45, which could have affected the concentration of this homologue in the rat tissues. Therefore, the effect of administering a diet containing predominantly ubiquinone-50 on the concentrations of these two homologues in the rat was examined. The relative amounts of each homologue present in the intestines and liver were estimated after chromatographic separation on paper as described below.

The animals were killed by a sharp blow on the back of the head and neck 24 hr. after the last dose of ubiquinone or food, and the various tissues excised immediately. Those tissues not analysed right away were stored at −22° until they could be extracted.

Preparation of intestinal sacs and incubation procedure. The animals used for the preparation of intestinal sacs were starved for a few hours before killing. The intestines were excised and washed out with 0·9% NaCl and everted by the procedure of Wilson & Wiseman (1954). The lumen was then filled with bicarbonate buffer (Krebs & Henseleit, 1932) and tied off in appropriate lengths to form sacs for insertion into 150 ml. conical flasks. The sacs were just covered with buffer. The labelled substrate was added directly to the buffer medium as required.

In Expts. III, V and VI some carrier ubiquinone was added to the buffer in the form of an aqueous dispersion with crystalline bovine albumin (5 mg./ml.). This enables the ubiquinone to be adsorbed into the mucosal cells. The flasks were fitted with bungs carrying inlet and outlet tubes and connected to an O_2+CO_2 (95:5) gas supply. The outlet from the final flask in the gas train was connected to a vessel containing 10% KOH solution to trap any $^{14}CO_2$ or ^{14}C-labelled volatile acids evolved. The flasks were then incubated at 37° in a water bath for the appropriate time with constant shaking at 120 excursions/min. At the end of the incubation the sacs were lifted out and carefully rinsed internally and externally. The suspension medium and washings were then centrifuged at 20 000 *g* for 20 min. to harvest any loose mucosal cells, which were added to the unbroken intestinal tissue for extraction.

Extraction of ubiquinone from tissues. The respective tissues from each set of animals were then saponified in the presence of pyrogallol as described by Mervyn & Morton (1959). The unsaponifiable matter (with carrier ubiquinone in certain experiments) was chromatographed on partially deactivated acid-washed alumina (Brockmann grade 3 prepared from grade O, Peter Spence and Co. Ltd., Widnes, Lancs.). The ubiquinone is eluted with 4–6% of ether in light petroleum (b.p. 40–60°). Occasionally a second chromatography was carried out on a 10 g. column of Celite–silicic acid (1:2, w/w) to purify the ubiquinone further. The eluting solvent in this case was 40–60% benzene in light petroleum. It was then possible to crystallize the ubiquinone from the minimum quantity of ethanol at 0° and then to recrystallize at room temperature.

In Expt. VIII it was more important from the analytical point of view to have all the ubiquinone from a tissue in

Table 1. *Recovery of ubiquinones after paper chromatography*

Preparations were placed as a 4 in. band at the starting line on Whatman no. 3 MM paper coated with petroleum jelly. Developing solvent was *NN*-dimethylformamide containing 2% of water. Ubiquinone zones were eluted with spectroscopically pure ethanol, and the eluate was made up to known volume. Uq, Ubiquinone.

	Ubiquinone					
	Applied		Recovered			
			Uq-45		Uq-50	
(*a*) Synthetic mixtures	Uq-45 (μg.)	Uq-50 (μg.)	(μg.)	(%)	(μg.)	(%)
1	400	131	321	80	100	79
2	400	66	314	79	51	77
3	400	131	336	84	103	76
4	400	66	345	86	57	87
			Mean	82	Mean	80

(*b*) Tissue fractions	Total (μg.)	Uq-45 (μg.)	Uq-50 (μg.)	Uq-45 + Uq-50 (%)
1	611	419	56	78
2	702	470	45	74
3	890	590	60	73
4	910	497	188	75
5	800	457	132	74
6	1513	632	575	80
			Mean	76

one fraction rather than have a smaller amount of purer material. In this case the ubiquinone was eluted from the alumina column in one fraction with 5% of ether in light petroleum.

Paper chromatography. The ascending reversed-phase system of Linn *et al.* (1959) was followed. Whatman paper no. 1 was used for the qualitative analysis and no. 3MM for the quantitative analysis. The paper fibres are coated with petroleum jelly as the stationary phase by dipping the rolled sheet in a 5% solution in light petroleum and allowing it to drain and dry uniformly. For the quantitative work the petroleum jelly is previously purified by passing a solution in light petroleum through a large column of alumina grade O (100 g.). This removed an impurity which absorbs very strongly in the 260 mμ region and would interfere with the spectrophotometric assay of the separated ubiquinones when eluted from the paper. A tiny drop of the ubiquinone solution is applied to the paper at the starting point for the qualitative work, but in the quantitative experiments the solution is applied as a narrow band about 4 in. long at the starting line by using a micropipette. The mobile phase is *NN*-dimethylformamide containing 2% of water. The spots or zones can be located by ultraviolet light and cut out. The ubiquinones are then eluted with spectroscopically pure ethanol into tubes kept in a closed box. The eluate, usually less than 5 ml., can be made up to a suitable volume for examination in the spectrophotometer.

The results of analyses of (*a*) synthetic mixtures and (*b*) fractions of liver unsaponifiable matter by this procedure are given in Table 1. They show that the recovery is only about 80%. The apparently slightly lower figures for liver fractions are due to the fact that these extracts also contain some ubiquinone-40 and ubiquinone-35 which are not included. The percentage recovery is much the same for both ubiquinone-45 and -50.

Estimation of ubiquinone. The amounts of ubiquinone in the chromatographic fractions were determined spectrophotometrically after correction for irrelevant absorption by the Morton & Stubbs (1946) procedure which has been found highly reliable for chromatography fractions containing not less than 20% of ubiquinone. The extinctions (E) of the extracts in *cyclo*hexane were read at the following three wavelengths: 262 mμ, (E_1), 272 mμ (E_2) and 282 mμ (E_3). The equation obtained was

$$E_{corr.} \text{ at } 272\ m\mu\ (\lambda_{max.}) = 2{\cdot}28(2E_2 - E_1 - E_3).$$

The constant 2·28 was derived from the absorption curve of the pure substance determined on the same spectrophotometer as that used in the routine analyses.

Where irrelevant end-absorption was strong but the maximum at 272 mμ was still clear-cut, use was made of the procedure described by Pumphrey & Redfearn (1960). The drop in $E_{275\ m\mu}$ was measured on the addition of a crystal of the reductant $NaBH_4$ to the ubiquinone solution in ethanol. The $\Delta E^{1\%}_{1\ cm.}$ value for pure ubiquinone-50 at 275 mμ was 142; and for pure ubiquinone-45 ΔE was 154, after allowing for the smaller molecular weight.

Radioassay. Suitable samples of all samples were applied to tared flat stainless-steel planchets of 1 in. diam., previously fitted with a disk of lens tissue (0·75 in. diam., Green's no. 105) and counted in the proportional region in a hemispherical gas-flow counter (Nuclear Measurements Corp., Chicago, U.S.A.) to $< 4\%$ probable error. All determinations were corrected for background and self-absorption (to zero thickness) in the usual way.

RESULTS

Biosynthesis. The various specimens of [^{14}C]-ubiquinone isolated with carrier ubiquinone-50 from the intestines, skin and livers of rats given [2-^{14}C]acetate either intraperitoneally or orally, were radioactive. The radioactivity persisted even after the second recrystallization. The specific activities corrected for dilution with carrier are given in Table 2. These give a general indication of the radioactivity of the synthesized ubiquinone fraction in each tissue, but the values for the individual homologues may be slightly different. The $E^{1\%}_{1\ cm.}$ values at 272 mμ for the crystalline preparations isolated were in good agreement with the expected value for the carrier ubiquinone-50 used. It is evident from Table 2 that the [2-^{14}C]acetate was incorporated into the ubiquinone of each tissue to different extents depending on the method of administration of the substrate. The specific activities of the pure cholesterol obtained from the same tissues (E. I. Mercer & J. Glover, unpublished work) are also inserted. The specific activity of the ubiquinone from each tissue is in general greater than that of the corresponding sterol, but the metabolic pool of cholesterol is very much greater.

The results also show clearly that oral administration of the radioactive acetate yields the labelled terpenoid compounds with a much higher specific activity in the intestine.

Table 2. *Incorporation of* [2-^{14}C]*acetate into ubiquinone by various tissues of the rat* in vivo

Expt. no.	I	II
Number of rats	3	3
Method of administration	Intraperitoneally	Orally
Total dose of [2-^{14}C]acetate (μc) ...	93·6	98·4
Dose/rat/day (μc)	10·4	16·4

	Specific activities of terpenoid compounds (counts/min./mg.)			Specific activities of terpenoid compounds (counts/min./mg.)		
Tissue	Ubiquinone	Cholesterol	Ratio	Ubiquinone	Cholesterol	Ratio
Intestine	1075	1420	0·8	30 420	23 100	1·3
Skin	1150	537	2·1	800	548	1·5
Liver	485	327	1·5	2 000	—	—

Neither of the above methods of administration completely ruled out the possible participation of intestinal flora in the biosynthesis so a number of experiments were carried out with everted intestinal sacs *in vitro*. The latter were incubated aerobically with [2-^{14}C]mevalonate in Krebs–Henseleit buffer for 3–9 hr. in the presence of ubiquinone as trapping agent for [^{14}C]ubiquinone. The tissue ubiquinone and carrier were then isolated and purified chromatographically. The ubiquinone fraction was radioactive, so it was crystallized several times to constant specific activity. The Expts. I–III, with ubiquinone-50 as carrier, were done before it was discovered that ubiquinone-45 is the main homologue in the rat. The drop in radioactivity between the second and third crystallizations was attributed to loss of ubiquinone-45 from the crystallization of the ubiquinone-50 carrier. The later experiments were done with ubiquinone-45 as carrier and no such loss of radioactivity occurred after three crystallizations. The results are given in Table 3.

In two experiments, however, where ubiquinone carrier was not added at the outset, the reisolated ubiquinone was unlabelled, which agrees with the findings of Wiss & Gloor (1960) and Phillips (1960) with rat-liver homogenates. The effect of adding the ubiquinone carrier at the outset of the experiment appears to be significant, although the activity trapped (200 counts/min./mg. or a total of 2×10^{-3} μc) is extremely small in comparison with the amount of [2-^{14}C]mevalonate (10 μc) used. It represents an incorporation of 0·02 %.

Thus it appears that isolated intestinal tissue can synthesize not only the side chain from small intermediary metabolites such as acetate and mevalonate but can also couple it to the nucleus, the source of which is still unknown.

Location of radioactivity in [^{14}C]*ubiquinone*. In the course of these experiments and parallel work on the absorption of dietary ubiquinone, it was discovered that normal rat liver and intestine contained traces of ubiquinone-40 and -35 respectively in addition to the -45 and -50 homologues previously reported (Rüegg *et al.* 1959; Linn *et al.* 1959). A mixed [^{14}C]ubiquinone specimen diluted with ubiquinone-50 was acetylated and degraded by ozonolysis as described by Morton *et al.* (1958), and the products were oxidized further to form laevulinic acid and the ring acid which contains the first two carbon atoms of the side chain.

The laevulinic acid was resolved from the ring acid and isolated as the crystalline 2:4-dinitrophenylhydrazone derivative (Rilling & Bloch, 1959). An attempt was made to obtain the aromatic nucleus in a pure form as the *S*-benzyl-*iso*thiouronium salt, but the yield was poor and the substance was not easily crystallized. The laevulinic acid carried the bulk of the activity at 46 000 counts/min./m-mole, whereas the ring compound possessed only 650 counts/min./m-mole, confirming that the main incorporation of radiocarbon occurred in the side chain.

Storage. From the relatively uniform values reported in the literature for the concentration of ubiquinone in various animal tissues, it appears that large reserves are not accumulated in the liver, unlike vitamin A. The capacity of rat tissues to retain even a temporary reserve was examined in a preliminary experiment VIII, the scale of which was limited by the amount of crystalline ubiquinone-45 available at the time. The ubiquinone contents of various tissues from the group of four dosed females killed 24 hr. after dosing are compared with those of the control group in Table 4. Only the liver and intestine of the dosed group contained amounts of ubiquinone-45 in excess of the normal. This suggested that a small reserve may be held but the values for the heart and kidney were unexpectedly a little lower in the test than in the control group. The variation is perhaps due to losses in purifying such small amounts. Of the 4·7 mg. administered to each rat only 0·6 mg. or 13 % of the dose was recovered from the tissues

Table 3. *Incorporation of* [2-^{14}C]*mevalonate into ubiquinone by everted intestinal sacs of the rat*

In some experiments carrier ubiquinone was added at the outset to the incubation medium of Krebs–Henseleit bicarbonate buffer containing 5 mg. of crystalline bovine albumin/ml. It was invariably necessary to add additional carrier (about 5 mg.) for the final crystallizations. Uq, Ubiquinone.

Expt. no.	III	IV	V	VI	VII
No. and sex of rats	6 F	3 M	6 M	6 M	6 M
Incubation period (hr.) ...	3	3	9	9	9
[2-^{14}C]Mevalonate added (μc)	9	13·6	10·6	12·5	12·5
Ubiquinone carrier (mg.) ...	6·7 (Uq-50)	0	9·8 (Uq-45)	5·8 (Uq-45)	0
	Specific activity of [^{14}C]ubiquinone isolated (counts/min./mg.)				
Chromatography fraction	2270	2667	3800	1960	1456
After 2nd crystallization	573	0	—	—	0
After 3rd crystallization	208	—	198	158	—
After 4th crystallization	—	—	214	172	—

24 hr. later. Thus the degree of absorption is poor or else its rate of metabolism and excretion is high. Koniuszy, Gale, Page & Folkers (1960), however, have reported that small amounts of ubiquinone are excreted in human urine.

The various ubiquinone fractions from the tissues of the above rats were examined qualitatively by reversed-phase paper chromatography. Ubiquinone-45 and -50 were observed in every tissue, with the former in considerable excess. In addition, small amounts of ubiquinone-40 and -35 were detected in fractions from liver, kidney and intestine, but not in the heart or skeletal-muscle preparations. It is possible, however, that, since only small quantities of the latter extracts were available for analysis, ubiquinone-40 and -35 concentrations were below the limit of detection.

A quantitative analysis was carried out on the liver ubiquinones of a group of three female rats (about 2 months old) maintained on the stock cube diet. Out of 611 μg. applied to the paper the relative proportions of the three major components estimated, ubiquinone-45, -50 and -40, were 419, 56 and 27 μg./liver (av. wt. 5·7 g.) respectively. Ubiquinone-35 was present at a lower level than ubiquinone-40. The recovery of ubiquinone in this analysis was about average (82%).

Origin of ubiquinone homologues. In order to check whether the ubiquinone-40 and -35 encountered in rat tissue came from the diet, 10 g. of lipid extract (from approx. 280 g. of powdered food cubes) were saponified for 30 min. in the presence of pyrogallol and in a nitrogen atmosphere. The ubiquinones were isolated from the unsaponifiable matter as described above. The content of total ubiquinones was 0·44 mg./100 g. of diet (uncorrected for slight destruction in the saponification of the lipids). The main component was the -45 homologue with only a little -50. The others were not detected so that the ubiquinone-40 and -35 were synthesized either by the microflora of the intestine or in the rat tissues.

The effect of changing the diet on the ubiquinone pattern in the tissues was examined next. In Expt. IX, five groups of animals were examined at weekly intervals after being placed on diet no. 2 enriched with ubiquinone-50. The proportions of ubiquinone-45 to -50 were measured in the livers of each group and compared with the corresponding values for controls. The results are given in Table 5. It became apparent as the experiment progressed that the administered ubiquinone-50 was not affecting the level of ubiquinone-45 in the tissues appreciably so the final group 5 was given supplements of 0·33 mg. of ubiquinone-50/rat/day for 3 days before being killed. It is clear from the results that even this relatively high concentration of ubiquinone-50 did not affect the endogenous ubiquinone-45 which tended to remain constant around 130 μg./g. of tissue. Similarly, in the intestine (where the ubiquinone-45 might have been expected to exchange more easily), the same situation was obtained. The values for total ubiquinones in intestinal tissue are given in Table 6, but the proportion of ubiquinone-45 to -50 was only measured in the last group during the period when ubiquinone-50 intake was optimum. It is quite clear from the absolute values and the ratios found, namely 2·2 (experimental) as against 3·1 (control), that dietary ubiquinone-50 has not affected or replaced the endogenous ubiquinone-45. This does not necessarily mean, however, that a metabolic fragment such as the nucleus of ubiquinone-50 may not be used for the biosynthesis of ubiquinone-45.

DISCUSSION

Although the total amounts of [^{14}C]acetate administered by different routes to the two groups of rats *in vivo* were approximately the same, the specific activities of the [^{14}C]ubiquinone isolated from the corresponding organs of the two groups differed widely. The organ or tissue which first received the labelled precursor in highest concentration produced ubiquinone with the greatest specific activity. The intestine had the ubiquinone with highest specific activity in both experiments, reflecting its high rate of metabolism. It is

Table 4. *Absorption of single doses of ubiquinone-45 by the rat*

Expt. VIII: nine female rats from the same litter were divided into two groups of five (controls) and four (test). Ubiquinone-45 (4·7 mg.) was administered as a single dose in arachis oil to each rat in the test group, 24 hr. before it was killed.

	Ubiquinone in controls		Ubiquinone in dosed		
Tissue	(μg./organ) (*a*)	(μg./g.) (*b*)	(μg./organ) (*c*)	(μg./g.) (*d*)	(*c* − *a*)
Intestine	103	18	205	39	+102
Liver	918	133	1490	225	+572
Heart	117	189	96	167	−21
Kidney	96	79	67	57	−29
Lung	7	9	5	7	−2
Skeletal muscle	—	27	—	18	—

Table 5. *Storage of dietary ubiquinone-50 in rat liver without any marked effect on the endogenous ubiquinone-45*

Expt. IX: there were three female rats in each group. Uq, Ubiquinone.

Week no.	Liver wt. (g.)	Ubiquinone contents (μg./g.) Total	Uq-45*	Uq-50*	Ratio
Controls: Ubiquinone-deficient diet					
1	4·0	127	—	—	—
2	5·7	124	112	11	10·2
3	6·5	120	109	11	9·9
4	5·2	146	132	13	10·1
5	5·3	172	153	16	9·6
	Mean values (±S.E.M.):		127 (±20)	13	9·9
Experimental: Above diet with ubiquinone-50 added (1 mg./100 g. of diet)					
1	3·3	160	—	—	—
2	4·7	172	131	38	3·4
3	5·7	160	116	44	2·6
4	4·0	217	158	59	2·7
5†	5·8	260	136	124	1·1
	Mean values (±S.E.M.):		135 (±17)	—	1·7

* These values are corrected for the slight loss of material in the chromatographic separation.
† Each rat in group 5 was given a supplement of ubiquinone-50 (1 mg./day) for 3 days before it was killed.

Table 6. *Ubiquinone in the intestines of rats on a diet enriched with ubiquinone-50*

Expt. X: there were three female rats in each group; the enriched diet contained 1 mg. of ubiquinone-50/100 g. of diet. Each rat in the last experimental group was given a supplement of ubiquinone-50 (1 mg./day) for 3 days before examination. Uq, Ubiquinone.

Week	Controls: Ubiquinone-deficient diet: Intestine wt. (g.)	Total ubiquinones (μg./g.)	Experimental: Uq-50-enriched diet: Intestine wt. (g.)	Total ubiquinones (μg./g.)
1st	15	30	10	32
2nd	14	31	14	37
3rd	12	24	16	22
4th	15	31	13	32
5th*	14	40	17	40
	Mean	31±6	Mean	33±7

* 5th, ratio Uq-45/Uq-50 = 3·1 (controls), 2·2 (experimental).

significant that the values run fairly parallel to those for the pure cholesterol from the same tissue. Together these findings lead to the conclusion that the side chain at least must have been elaborated in each tissue, and not in one organ alone.

Since the metabolic pool of cholesterol is very much larger than that of ubiquinone (about 100 times), the total radioactivity incorporated into the sterols was very much greater, in agreement with the findings of Gloor & Wiss (1959*a*). The turnover rate of the isoprenoid side chain of ubiquinone must therefore be very much slower than that of cholesterol. Presumably it is elaborated in the form of the pyrophosphate in a manner similar to that for farnesyl pyrophosphate and nerolidyl pyrophosphate (Lynen, Eggerer, Henning & Kessel, 1958; Goodman & Popják, 1960). If it is formed from mevalonate almost as rapidly as the latter pyrophosphates then the slow turnover rate indicates that the final stages of ubiquinone biosynthesis, such as the coupling of the side chain to the nucleus, are probably rate-limiting. The work of Gloor & Wiss (1959*a*) indicates that the preformed nucleus in the form of 3:4-dimethoxy-2:5-toluquinone cannot be used for the biosynthesis so some modified structure must be found.

The incorporation of the small but significant amount of [2-^{14}C]mevalonate into intestinal ubiquinone *in vitro* when carrier material is added whereas none is found in its absence requires comment. The difference between the results can hardly be attributed to microbiological synthesis even though the incubation period was 9 hr. in some cases, for bacteria usually elaborate the -30 and -35 homologues. A small amount of the nucleus precursor should be present in the tissue when it is

excised from the animal so that the newly synthesized ^{14}C-labelled side chain may become coupled to it. The carrier ubiquinone added at the outset may merely have a protecting or sparing effect on this new material during the incubation period. Alternatively, it could perhaps on metabolism provide some of the nuclear moiety required for the biosynthesis of new ubiquinone. The turnover of ubiquinone *in vivo* appears relatively slow from Expts. I and II: therefore one cannot expect rapid incorporation of radioactivity *in vitro*. The solution of the problem may be obtained by using ubiquinone labelled with ^{14}C in the ring. The experiment conducted by Rudney & Sugimura (1960) on the administration to rats of [^{14}C]ubiquinone-30 prepared from yeast grown in the presence of sodium [^{14}C]formate is inconclusive. These authors reported that the nucleus of ubiquinone-30 could not provide the nucleus of ubiquinone-50. This material presumably carries the ^{14}C on the methoxyl group and this may be quite labile as suggested by recent evidence in this Laboratory with [*methyl*-^{14}C]methionine. The methyl group of methionine can provide the methoxyl-methyl of ubiquinone in the rat and may be transferred by an *O*-methyl transferase (Lawson & Glover, 1961). It would appear that a series of 'activated' polyisoprenoid units are normally present in rat tissues as intermediates in the biosynthesis of terpenoid compounds such as the sterols, side chains of vitamins E and K, ubiquinones and ubichromenol, and dolichol (Pennock, Hemming & Morton, 1960). The size of side chain coupled to the quinone nucleus is probably controlled by the specificity of the particular enzyme in the organism under study.

The results for the administration of ubiquinone-45 and -50 to rats (Tables 4 and 5) indicate that a little storage of ubiquinone takes place in the liver but probably at a site different from that of the endogenous ubiquinone-45, since the concentration of the latter is not appreciably affected by the administration of ubiquinone-50 (see Table 5). Yet during this period of 5 weeks the level of ubiquinone-50 in the liver rose steadily from an average level of 13 μg./g. to 124 μg./g. In the intestine, the ubiquinone-45 level also remains very steady despite the transit of much more ubiquinone-50 across the gut wall. These observations lead to the conclusion that dietary ubiquinone-50 does not easily replace the endogenous material, which is found mainly in the mitochondria (Hemming, Pennock & Morton 1958) and it may be stored elsewhere in the cell.

The ratio of ubiquinone-45 to -50 in the livers of the control rats on the synthetic diet (low in ubiquinones) was approximately 9·9:1, which is a little higher than that found by Gloor & Wiss (1959*b*, 1960) but much higher than the values 5·0–5·6 observed by Olson & Dialameh (1960) and 2·2–3·0 obtained by Wiss & Gloor (1960) for normal and vitamin A-deficient rats. Our own stock-colony rats on the food cubes known to contain mainly ubiquinone-45 show a ratio in the region of 7·5:1. These differences should probably be attributed more to dietary effects than to intestinal flora or variations of strain.

In the control groups of rats (see Tables 5 and 6) the ratio for the liver is quite different from the ratio (between 2:1 and 3:1) for the intestine.

Possibly each tissue manufactures its own characteristic proportion as it is known to do with the sterols. Unlike the latter, however, dietary ubiquinone does not appear to affect the synthesis of the endogenous material.

Attention should perhaps be turned to smaller molecules as potential sources of the ring nucleus of ubiquinone. Whereas the ubiquinone molecule appears to be assembled mainly within the tissue cell, the quinone nucleus may be obtained from an outside source.

SUMMARY

1. The isoprenoid side chain of ubiquinone can be synthesized from acetate by each tissue of the rat *in vivo*. The rate of incorporation of [2-^{14}C]-acetate into ubiquinone, however, is much slower than into cholesterol.

2. Small but significant amounts of [2-^{14}C]-mevalonate can be incorporated into ubiquinone by the everted intestinal sac in the presence of unlabelled ubiquinone supplements. A small quantity of aromatic nucleus is apparently available in the tissue under these conditions.

3. The tissues of normal stock-colony rats contain mainly ubiquinone-45 with smaller amounts of the -50, -40 and -35 homologues in that descending order. The relative proportions in the liver are affected by dietary ubiquinones, which can be stored there temporarily.

4. The administration of ubiquinone-50, however, in amounts from 100 to 450 μg./rat/day did not affect the concentrations of ubiquinone-45 in liver and intestine, which tend to remain constant at about 130 and 32 μg./g. of tissue respectively. It appears therefore that the exogenous ubiquinone cannot exchange with or flush out the endogenous material which is in the mitochondria. It is presumably localized in another part of the cell and may therefore not be functional in the animal itself.

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